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PRINCIPAL INVESTIGATOR: Robert B. Dickson, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20007

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Robert B. Dickson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20007

E-Mail: dickson@georgetown.edu

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13. ABSTRACT (Maximum 200 Words)

Many clinical studies have shown that apoptosis may be related to various pathological parameters of breast cancer, such as tumor size, histologic features, metastasis, and survival. Over 50% of human breast cancer biopsies show amplification or overexpression of c-myc, an oncogene that is known to play a crucial role in cell proliferation, apoptosis, and transformation. Female c-myc transgenic mice also develop mammary cancer that is characterized by a large number of apoptotic cells, thus serving as a good in vivo model for study on the role of c-Myc in both mammary carcinogenesis and apoptosis. On the other hand, TGFa, a growth factor also frequently overexpressed in human breast cancer, has been shown in MT-tgfa/MMTV-c-myc double transgenic mice to enhance c-Myc-induced mouse mammary carcinogenesis, probably in part by blocking apoptosis. Our proposal set out to examine the c-Myc mechanisms of mediated apoptosis in our mouse mammary tumor model. In addition, we examined the survival-promototing effect of the TGFa-EGF receptor pathway in this model. We initially provided evidence for TGFa-EGF receptor emediated cell survival by a calcium/calmodulin mediated pathway regulating Akt. Subsequent studies have focused on the role of a recently discovered kinase, PNCK, and P55g, a pI3 kinase subunit to mediate the EGF survival signal.

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Introduction

Apoptosis is a type of cell death that has been programmed in a cell for its elimination, when it is no longer needed. Many clinical studies have shown that programmed cell death may be related to various biologic parameters of breast cancer, such as tumor size, morphologic features, metastasis, and survival. Over 50% of human breast cancer biopsies show amplification or overexpression of *c-myc*, an oncogene that is known to play a crucial role in cell proliferation, apoptosis, and malignant transformation. Mice carrying a *c-myc* transgene also develop mammary cancer that is characterized by a large number of apoptosis cells. These data suggest that *c-myc* may play an important role in the breast cancer formation and in apoptosis of the cancer cells and that the *c-myc* transgenic mouse may serve as a good *in vivo* model for the study of these aspects of breast cancer. On the other hand, TGFα, a growth factor also frequently overexpressed in human breast cancer, has been shown in *tgfα/c-myc* double transgenic mice to enhance c-Myc-induced mammary cancer development, probably in part by blocking apoptosis. Our initial data showed that in *c-myc* transgenic mammary tumors, several apoptosis-promoting proteins, including cytochrome c (Cyt-c), Apaf-1, and caspase-9, that form a complex termed the apoptosome, were expressed mainly in the apoptotic cells. However, caspase-3, one of 3 enzymes that executes apoptosis, upon activation by the apoptosome, was not expressed at detectable levels in the apoptotic cells. On the other hand, AIF, which does not need to be activated by caspase to induce apoptosis, was found in the nuclei of apoptotic cells. *We initially hypothesized* that c-Myc-induced apoptosis in *c-myc* transgenic mammary tumors may involve AIF and the apoptosome, but not the executor caspases. Our preliminary data also showed that caspase-8, a mediator of several extracellular signals for apoptosis, was abundantly expressed and present as both activated and inactivated forms. However, it was localized mainly to the major tumor areas that were not apoptotic. We therefore had hypothesized that in *c-myc* transgenic mammary tumors, c-Myc-induced apoptosis may involve activation of caspase-8, but in most c-Myc-expressing, non-apoptotic cells, this pathway is blocked somewhere downstream of caspase-8. The caspase-8-mediated and the apoptosome-mediated pathways may cross-talk with each other, possibly *via* a link between Cyt-c and caspase-8. Moreover, since apoptotic cells rarely appeared in mammary tumors from *tgfα/c-myc* double transgenic mice, we had also initially hypothesized that, in the double transgenic mammary tumors, TGFα blocks c-Myc-induced apoptosis by inhibition of activation of certain caspase-independent factors, such as AIF, and/or by inhibition of the factors that block the downstream events of caspase-8 and/or of the apoptosome.

In our proposed studies, we were to conduct a dual approach to understanding the mechanisms of c-Myc-induced apoptosis and TGFα - induced survival; first, *in vitro*, using our cell line models. Next, we had proposed to utilize an *in vivo* transgenic tumor model, to further validate results. Specifically, in the first Aim, we were to examine the effect of EGF withdrawal from our c-Myc expressing mouse mammary tumor model on apoptosis, dependent upon either AIF or executor caspases. We would then follow-up any *in vitro* positive results, showing executor caspase-independent apoptosis with *in vivo* testing. In the second Aim, we would evaluate the possible role of caspase 8 *in vitro*, followed by *in vivo* studies if caspase 8 were involved. In the final Aim, we would explore the mechanism of EGF/TGFα promoted survival of c-Myc-expressing, pro-apoptotic cells. However, for Aims 1 and 2, we found that, contrary to our hypothesis, c-Myc promoted apoptosis depended upon executor caspases but not caspase 8. In addition, for Aim 3, we made novel observations implicating Ca++/ calmodulin in activation of Akt, to mediate EGF/TGFα-promoted survival. We proposed and had approved a revised statement of work to further examine these novel findings, with special reference to pregnancy-upregulated non-ubiquitus CaM kinase (Pnck).

Body

In the proposed grant, we were to address the following Statement of Work:

Task 1: Determine whether c-Myc-induced apoptosis in *c-myc* transgenic mammary tumors involves AIF and the apoptosome (Cyt-c, Apaf-1, and caspase 9), but not the executor caspases – 3, -6, or –7.

Status: Negative results obtained, implicating executor caspases. Task completed.

Task 2: Determine whether caspase –8 is involved in c-Myc-induced apoptosis in *c-myc* transgenic mammary tumors and determine how it is related to the apoptosome-mediated pathway.

Status: Negative results obtained, ruling out caspase 8. Task completed.

Task 3: Study the mechanisms by which TGFα blocks the c-Myc-induced apoptosis in MT-*tgfα*/MMTV-*c-myc* double transgenic mammary tumors.

Status: Positive results, implicating Akt in the EGF receptor-mediated survival pathway, with the unexpected implication of Ca++/calmodulin signaling in the process. Revised Statement of Work [below] was approved by the DOD to capitalize on these findings.

Subtask 3a: Determine, using PNCK gene transfection into mouse mammary epithelial cells, whether this kinase functions primarily to block proliferation or to promote apoptosis.

Subtask 3b: Determine, using yeast 2-hybrid methodology what are major substrates of PNCK. We will then compare these substrates to our existing c-Myc expression database for design of further work to explore anti-tumor mechanisms of PNCK in mammary cancer.

Status: Revised subaims in progress.

Task 1: In Task 1, we began by studying the possible role of executor caspases in c-Myc-induced apoptosis. Although we had not expected involvement of executor caspases, we found that EGF withdrawal from our Myc 83 (c-Myc expressing cell model) induced cleavage of PARP, an executor caspase substrate. This cleavage was blocked by treatment of cells with z-VAD-funk, a broad specificity caspase inhibitor. Based on these results, we concluded that the underlying hypothesis of Task 1 was incorrect, and that there was nothing particularly unusual about c-Myc-induced apoptosis, relative to executor caspase utilization. We therefore deprioritized the *in vivo* part of the task, and moved on to Task 2.

Task 2: In Task 2, we employed a specific inhibitor of caspase 8, z-LETD-funk, to test the involvement of caspase 8 in the apoptosis induced by EGF withdrawal of Myc 83 cells. Results were again negative, indicating no involvement of caspase 8. However, when cells were treated with FASL, a known, proapoptotic inducer of caspase 8, apoptosis was inhibited, as expected, by z-LETD-funk. Accordingly, the hypothesis underlying Task 2 was shown to be incorrect, and we again de-prioritized *in vivo* follow-up experiments, moving on to Task 3.

Task 3: In Task 3, we initially set out to study survival mechanisms, engaged by EGF/TGFα treatment of c-Myc-expressing mouse mammary tumor cells. We completed a study demonstrating that EGF receptor activation activates AKT and upregulates $BclX_L$. Pharmacologic inhibition of PI3K/AKT and MEK/Erk signaling pathways partially inhibited induction of apoptosis, in association with downregulation of Akt and Erk1/2 protein levels. We also used a constitutively activated Akt (myr-Akt) to inhibit apoptosis in the same system, confirming a survival-promoting role of Akt. To further address the mechanism of EGF receptor-dependent activation of Akt, we decide to explore the possibility of PI3-K and MEK/Erk – independent

pathways of activation. This objective was pursued by testing of a variety of common signal transduction inhibitors on our EGF-promoted, Myc 83 cell survival model. Surprisingly, we found that EGF-mediated Akt activation (and cell survival) was nearly abolished by calcium chelation and by inhibitors of Ca++/calmodulin.

Calcium ion, an important intracellular signaling molecule, exerts may of its signaling properties by interaction with a calcium binding protein called calmodulin. Calmodulin, coupled with calcium is able to interact with and activate a variety of kinases, termed calcium-calmodulin dependent protein kinases (CaM kinase). CaM kinases are extensively studied in the nervous system, where they play a critical role in intra-neuronal signaling, and in muscular tissue, where many of their substrates have been identified including cytokeletal proteins, such as actin and myosin. Their role in mammary epithelium signaling was virtually unknown, until a few years ago. Today, little is know about the role of CaM kinase in the breast. A few CaM kinases, however, have been discoved to be expressed in both normal and transformed mammary epithelial cells. One of these CaM kinases, CaM kinase III was demonstrated to be selectively active in proliferating mammary epithelial cells, with increased specific activity in fresh human breast tumor samples compared to adjacent normal tissue. Additionally, CaM kinase III is stimulated by EGF and IGF-1 in serum-starved breast cancer cells lines MCF-7 and MDA MB-231. When Myc83 cells were treated with Rottlerin, and inhibitor of CaM kinase III inhibitor and PKC delta, they lost Akt activation, even in the presence of EGF. Specific inhibitors of other CaM kinases had no effect on Akt activation in Myc83 cells, eliminating a role for PKC delta in the EGF signaling cascase in Myc83 cells. This data, taken together, suggests that Akt activation, and its subsequent survival signals are positively regulated, upstream, by CaM kinase III.

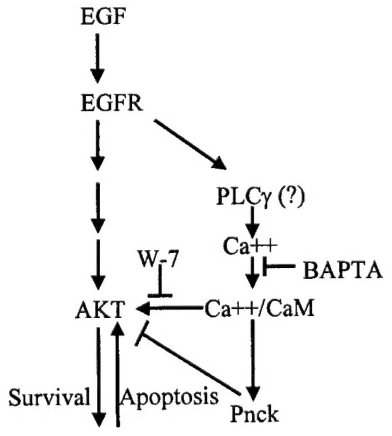
PNCK, a newly identified calcium/calmodulin kinase, is reported to be specifically expressed in MMTV-c-Myc cells (2, 3). Calcium and its ubiquitous sensor, calmodulin, are important intracellular signaling molecules that participate in a variety of cellular pathways including growth regulation, transcription and even neurotransmitter release. Our recent investigations revealed that calmodulin is overexpressed in epithelial cells derived from MMTV-c-Myc transgenic mouse mammary tumor (4). Previous studies from different laboratories, convincingly demonstrated that calcium is released from intracellular stores in response to growth factors in mammary epithelial/carcinoma cells. These observations, coupled with our observation of upregulation of calmodulin in MMTV-c-Myc cells, prompted us to investigate the role of calcium/calmodulin in cellular survival in MMTV-c-Myc cells. Calmodulin is known to positively regulate neutrophin/BDNF-induced survival in neuronal cells. The initial observations reported that Pnck is upregulated *in vitro*, in over-confluent and serum starved cells, compared to actively growing mammary epithelial cells. They suggest that Pnck expression is inversely related to mammary epithelial cell proliferation or may have a role as a negative regulator of cellular survival/proliferation. *In vivo*, Pnck is upregulated during certain stages of mammary gland development, specifically in late pregnancy and during post-lactational involution when epithelial cells are undergoing terminal differentiation, with decreased proliferation. We reasoned that before investigating the functional aspects of Pnck in mammary epithelial cell survival, we should first investigate the survival mechanism in these cells (Model 1). Accordingly, a series of investigations on survival signaling were carried out. MMTV-c-Myc cells were found to be sensitized to apoptosis in the absence of serum and growth factors. Addition of epidermal growth factor (EGF) rescued these apoptotic cells to survival from apoptosis (5). Further investigation on pro-survival nature of EGF revealed that it activates pro-survival kinase Akt, since constitutively active Akt (myr Akt) when overexpressed, also rescued apoptotic cells in absence of EGF (5). EGF-induced Akt activation was found to be PI-3 kinase (5), calcium and calmodulin dependent since LY294002 (PI-3 kinase inhibitor), BAPTA-AM (calcium chelator) and W-7 (calmodulin antagonist) all inhibited EGF-induced Akt activation (Fig.1). Biologically apoptosis could be induced by LY294002 (5) and by W-7 in MMTV-c-Myc cells, further implying that PI-3 kinase and calmodulin-mediated Akt activation ais an integral part of the cell survival mechanism (Fig.2).

In order to investigate whether calcium/calmodulin-mediated Akt activation occurs in other mammary epithelial cells, other non-tumorigenic human mammary epithelial cells, along with their c-Myc overexpressing counterparts were tested for W-7 sensitivity on EGF-induced Akt activation. In all of these cell lines tested, EGF-induced Akt activation was inhibited by W-7, implying that calmodulin may be a central regulator of

survival mechanismin mammary epithelial cells, irrespective of tumorigenicity, species, and c-Myc expression level (Fig.3A and B). Furthermore, Akt activation by other survival ligands, such as insulin, fetal bovine serum (FBS) were also inhibited by calmodulin antagonist W-7, indicating that other survival factors including EGF, also transduce survival signaling through calmodulin (Fig.3C).

We examined the possible mechanism(s) downstream of calmodulin and upstream of Akt in MMTV-c-Myc cells. Since, calmodulin kinases are the probable candidates, we employed a pharmacological inhibitor approach. STO-609 (CaM kinase kinase inhibitor), KN-62 (CaM kinase II inhibitor) and Rottlerin (CaM kinase III inhibitor) could not inhibit EGF-induced Akt activation. These results suggest that calmodulin mediated Akt activation is not mediated through any calmodulin kinase. Pnck has strong homology with CaM kinase I at the catalytic domain. STO-609 specifically inhibits CaM kinase kinase, which is an upstream activator of CaM kinase I. Since STO-609 has no effect on EGF-induced Akt activation, it strengthen the previous observation that Pnck is not a part of the cell survival machinery of MMTV-c-Myc cells.

Since EGF-induced Akt activation is connected to both PI-3 kinase and calmodulin in MMTV-c-Myc cells, and since calmodulin was previously reported to activate PI-3 kinase, we investigated whether these two events were interconnected. Calmodulin-sepharose specifically pulled down a non-ubiquitous regulatory subunit ($p55^{PIK}$) in a calcium dependent manner in an *in vitro* assay (Fig.4). Classical ubiquitous p85 regulatory sub unit was not detected in calmodulin-sepharose precipitates. The binding of calmodulin and p55PIK could be inhibited by EGTA. EGF-indiced Akt activation was inhibited by the PLC-γ inhibitor, U-73122. This observation suggests that an EGF-induced and calcium/calmodulin dependent PI-3 kinase activation leading to Akt activation, may be present in these cells. Neither the calmodulin antagonist W-7 nor U-73122 could inhibit binding of ubiquitous PI-3 kinase regulatory subunit, p85, to phosphotyrosine. *In vitro* PI-3 kinase assay further revealed that W-7 has no effect on EGF-induced PI-3 kinase activity associated with anti-phosphotyrosine and anti-p85 immunoprecipitates. At present time, we are examining how calmodulin-associated PI-3 kinase activity is affected by W-7. Our observations suggest that both classical phosphotyrosine / p85 dependent PI-3 kinase activity and a PLC-γ /calcium/calmodulin-$p55^{PIK}$ -mediated PI-3 kinase activity are operating, converging into AKT activation and survival of these c-Myc overexpressing breast cancer cells (Model 1). It will be interesting to examine how this positive, calmodulin-mediated survival signaling is modulated by overexpression of an apparent apoptotic kinase, Pnck. It is possible that Pnck-mediated-apoptotic signaling mechanisms counteract the calmodulin-mediated survival signaling/tumorigenesis. Pnck-mediated signaling mechanisms may be responsible for long latency of tumor development in MMTV-c-Myc mice. Our future investigations are thus directed to further understand Pnck-regulated signaling mechanisms.



Model 1: Possible role of Pnck in survival signaling

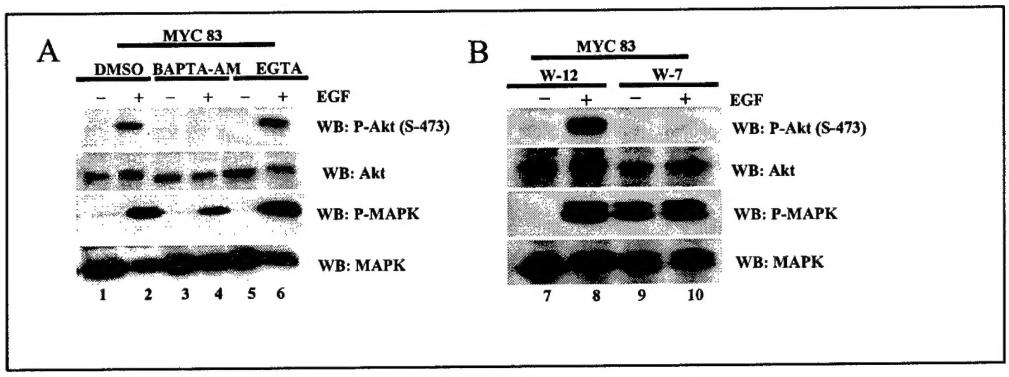


FIG.1 EGF-induced Akt activation is inhibited by calcium chelator BAPTA-AM and calmodulin antagonist, W-7. (A) Myc 83 cells (MMTV-c-Myc) were serum starved overnight and incubated with DMSO (lanes 1-2), 10 μM BAPTA-AM for 90 min (lanes 3-4) and 2mM EGTA (lanes 5-6) for 90 min followed by 3 minutes of EGF stimulation. Lysates were probed for activated Akt [WB: P-Akt (S-473)] and activated MAP kinase (WB: P-MAPK). Blots were reprobed for total Akt (WB: Akt) and total MAP kinase (WB: MAPK), respectively. (B) Serum starved cells were incubated with 30 μM W-7 or inactive analogue W-12 for 30 min. Cells were stimulated with 10 nM EGF for 3 min and processed for Akt and MAK kinase activation as described in Fig.1A

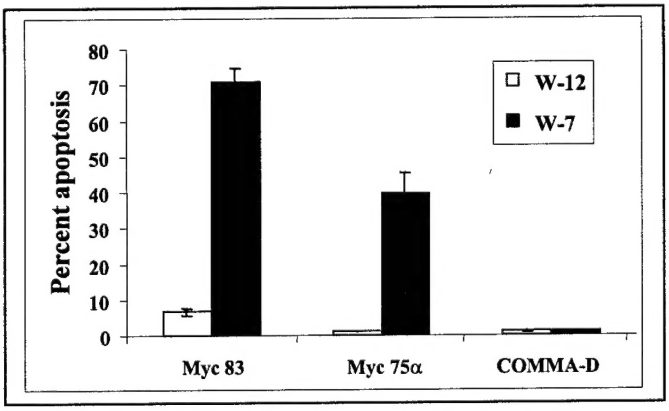


FIG.2 Calmodulin antagonist W-7 induces apoptosis in MMTV-c-Myc (Myc 83 cells) and MMTV-c-Myc/TGF-α (Myc 75 α) but not in Comma-D cells.
Semiconfluent growing cells were incubated with 30 μM W-12 or W-7 for 36 hours and all floating and adherent cells were stained with 10μg/ml of Hoechst 33258 dye (Sigma) for apoptotic analysis. For each replicate, at least 500 cells were counted and evaluated for the presence of condensed nuclei and overall apoptotic appearance. Each treatment was conducted in triplicate, and the experiment was repeated three times. A representative experiment is presented.

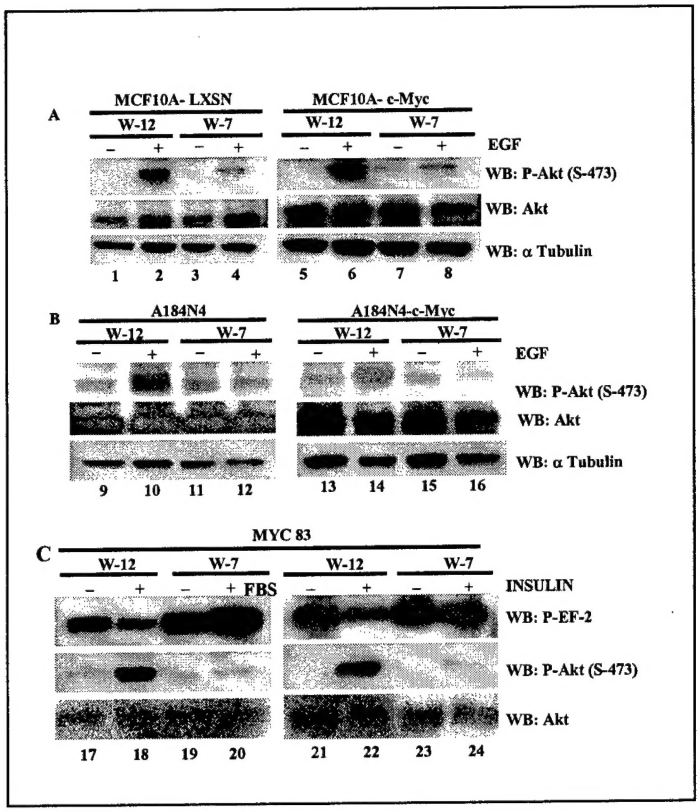


Fig. 3. Calmodulin mediates Akt activation in mammary epithelial cells, irrespective of tumorigenecity, species, c-Myc expression, and survival ligands. (A) MCF-10A-LXSN (lanes 1-4) and MCF-10 A-c-Myc (lanes 5-8) (B) A184N4 (lanes 9-12) and A184N4-c-Myc (lanes 13-16) cells and (C) Myc 83 cells were serum starved and incubated with 30 μM W-7 or W-12 for 30 min and stimulated either with or without 10 nM EGF for 3 min (A and B), 10% FBS for 5 min (lanes 17-20) and 100 nM insulin for 5 min (lanes 21-24) (C). Lysed cells were examined for Akt or EF-2 phosphorylation and total Akt. Blocking of EGF-induced dephosphorylation of EF-2 confirms the specificity of W-7.

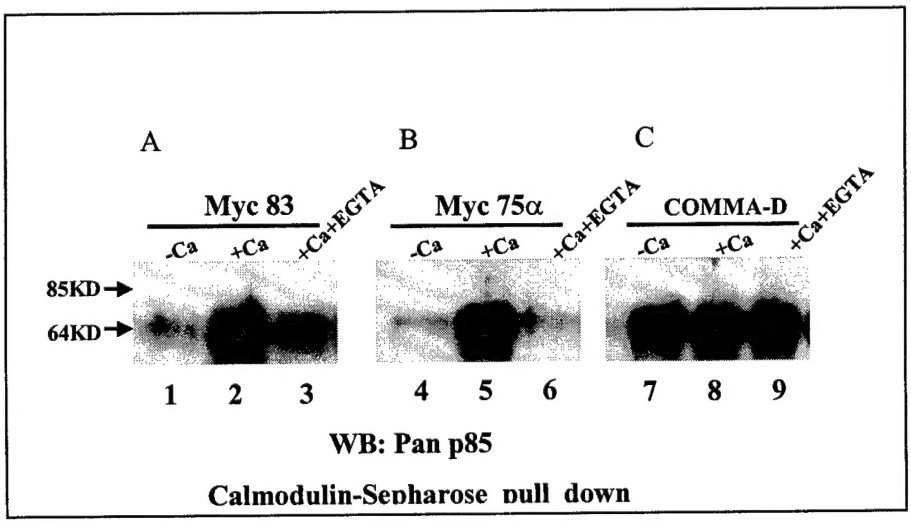


Fig.4. Calmodulin binds to $p55^{PIK}$/p55γ. Serum starved Myc 83 (A) Myc 75α (B) and Comma D (C) cells were lysed in EDTA-free lysis buffer, and 1 mg/ml total proteins were incubated with 30μl calmodulin-sepharose suspension for 2 h at 4 °C with or without 100 μM $CaCl_2$ (lanes 1-2, lanes 4-5, lanes 7-8). A third lysate from each cell line was incubated with 100 μM $CaCl_2$ in the presence of 1mM EGTA (lanes 3, 6 and 9). Calmodulin-sepharose beads were probed with a pan p85 antibody (p85α/β/$p55^{PIK}$/p55γ). Control-sepharose suspension did not precipitate $p55^{PIK}$/p55γ in calcium dependent manner (data not shown). $P55^{PIK}$/p55γ migrates with a eletrophoretic mobility of 64KDa on SDS-PAGE.

Key Research Accomplishments

1. Determination that c-Myc-promoted apoptosis in c-Myc-initiated transgenic mouse mammary tumor cells depends upon executor caspases, but not caspase 8.

2. Determination that EGF-mediated survival of c-Myc-initiated transgenic mouse mammary tumor cells is partially mediated by PI3-K/Akt and MEK/Erk pathways.

3. Discovery that EGF-mediated survival of c-Myc-initiated transgenic mouse mammary tumor cells is largely dependent upon Ca++/calmodulin signaling, potentially *via* PNCK, a recently discovered, c-Myc-regulated, Ca++/calmodolin-dependent protein kinase.

4. Establishment of a database (and publication of) c-Myc-induced genes in MMTV-c-Myc transgenic mouse mammary tumors, to serve as a resource for experiments to determine the possible survival-regulatory role of PNCK in this mammary tumor type.

5. Discovery of EGF/Ca++/calmodulin-mediated regulation of survival through induction of p55γ, a regulatory subunit of phosphatidyl-3-kinase.

Reportable Outcomes

Papers Published:

1. Desai KV, Xiao N, Wang W, Gang L, Greene J, Powell JI, Dickson RB, Furth P, Hunter K, Kucherlapati R, Simon R, Liu ET, and Green JE, Initiating oncogenic event determines gene expression patterns of human breast cancer models, Proc Nat'l Acad Sci (USA), 99:6967-6972, 2002.

2. Ramljak D, Coticchia C, Nishanian GT, Saji M, Ringel MD, Conzen SD, and Dickson RB; Epidermal growth factor inhibition of c-myc-mediated apoptosis through Akt and Erk involves Bcl-X_L upregulation, Exp. Cell. Res., 287:397-410, 2003.

3. Ramljak D and Dickson RB, Cell signaling in the breast, in Dennis and Bradshaw (eds), Handbook of Cell Signaling, Academic Press, 345:565-571, 2003.

Abstracts Published:

1. Ramljak D, Coticchia CM, and Dickson RB, Epidermal growth factor receptor signaling inhibits c-Myc-induced apoptosis through activation of Akt, Erk, and upregulation of BclXL in mouse mammary carcinoma cells, AACR Symposium on Apoptosis, Keystone Co, 2001.

2. Desai KV, Xiao N, Wang W, Gangi L, Greene J, Powell JI, Dickson RB, Furth P, Hunter K, Kurherlapati R, Simon R, Liu ET, and Green JE, Probing oncogenic pathways in transgenic mouse models of mammary cancer by cDNA microarray analysis, Proceedings of the AACR, San Francisco, CA, 2002.

3. Ramljak D, Coticchia CM, Nishanian TG, and Dickson RB, AKT inhibits c-Myc-mediated apoptosis in mammary epithelial cells: a mechanistic investigation, DOD ERA of Hope Meeting, Orlando, FL, 2002.

4. Deb TB, and Dickson RB, Calmodulin as a positive modulator of EGF survival signaling in MMTV-c-MYC mouse mammary epithelial cells, DOD ERA of Hope Meeting, Orlando, FL, 2002.

Conclusions

1. c-Myc-promoted apoptosis in c-Myc-initiated transgenic mouse mammary tumor cells depends upon executor caspases, but not caspase 8.

2. EGF-mediated survival of c-Myc-initiated transgenic mouse mammary tumor cells in partially mediated by PI3-K/Akt and MEK/Erk pathways.

3. EGF-mediated survival of c-Myc-initiated, transgenic mouse mammary tumor cells is largely dependent upon Ca++/calmodulin signaling, potentially *via* PNCK, a recently discovered, c-Myc-regulated Ca++/calmodolin-dependent protein kinase.

4. We also established a database (and publication) of c-Myc-induced genes in MMTV-c-Myc transgenic mouse mammary tumors to serve as a resource for experiments to determine the possible survival-regulatory role of PNCK in this mammary tumor type.

5. EGF-mediated survival of c-Myc initiated, mouse mammary tumor cells proceeds through induction of $p55^{PIK}$/p55γ, a regulatory subunit of phosphatidge-3-kinase.

References

1. T.G. Parmer, M.D. Ward, E.J. Yurkow, V. H. Vyas, T. J. Kearney, W. N. Hait (1999) Activity and regulation by growth factors of calmodulin-dependent protein kinase III (elongation factor 2-kinase) in human breast cancer. Br. J. Cancer, 79, 59-64.

2. H.P. Gardner, J.V. Rajan, S.I.Ha, N.C. Copeland, D.J. Gilbert, N.A. Jenkins, S. T. Marquis, L.A. Chodosh (2000) Cloning, characterization, and chromosomal localization of Pack, a Ca2+/calmodulin-dependent protein kinase. Genomics, 63: 279-288.

3. H. P. Gardener, S.I. HA, C. Renolds, L. A. Chodosh (2000) The CaM Kinase, Pnck, is spatially and temporally regulated during murine mammary gland development and may identify and epithelial cell subtype involved in breast cancer. Cancer Research, 60: 5571-5577.

4. Desai KV, Xiao N, Wang W, Gang L, Greene J, Powell JI, Dickson RB, Furth P, Hunter K, Kucherlapati R, Simon R, Liu ET, and Green JE, Initiating oncogenic event determines gene expression patterns of human breast cancer models, Proc Nat'l Acad Sci (USA), 99:6967-6972, 2002.

5. Ramljak D, Coticchia C, Nishanian GT, Saji M, Ringel MD, Conzen SD, and Dickson RB; Epidermal growth factor inhibition of c-myc-mediated apoptosis through Akt and Erk involves Bcl-X_L upregulation, Exp. Cell. Res., 287:397-410, 2003.

Appendix

1. Desai KV, Xiao N, Wang W, Gang L, Greene J, Powell JI, Dickson RB, Furth P, Hunter K, Kucherlapati R, Simon R, Liu ET, and Green JE, Initiating oncogenic event determines gene expression patterns of human breast cancer models, Proc Nat'l Acad Sci (USA), 99:6967-6972, 2002.

2. Ramljak D, Coticchia C, Nishanian GT, Saji M, Ringel MD, Conzen SD, and Dickson RB; Epidermal growth factor inhibition of c-myc-mediated apoptosis through Akt and Erk involves Bcl-X_L upregulation, Exp. Cell. Res., 287:397-410, 2003.

3. Ramljak D and Dickson RB, Cell signaling in the breast, in Dennis and Bradshaw (eds), Handbook of Cell Signaling, Academic Press, 345:565-571, 2003.

Initiating oncogenic event determines gene-expression patterns of human breast cancer models

Kartiki V. Desai*, Nianqing Xiao†, Weili Wang*, Lisa Gangi‡, John Greene§, John I. Powell§, Robert Dickson¶, Priscilla Furth¶, Kent Hunter‖, Raju Kucherlapati**, Richard Simon†, Edison T. Liu†††‡‡, and Jeffrey E. Green*†††§§

*Laboratory of Cell Regulation and Carcinogenesis, †Molecular Statistics and Bioinformatics, ††Advanced Technology Center, National Cancer Institute, Bethesda, MD 20892; ‡Microarray Core Facility, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD 21702; §Center for Information Technology, National Institutes of Health, Bethesda, MD 20892; ¶Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20007; ‖Laboratory of Population Genetics, National Institutes of Health, Bethesda, MD 20892; and **Harvard Partners Center for Genetics and Genomics, Boston, MA 02115

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Molecular expression profiling of tumors initiated by transgenic overexpression of *c-myc*, *c-neu*, *c-ha-ras*, polyoma middle T antigen (*PyMT*) or simian virus 40 T/t antigen (T-ag) targeted to the mouse mammary gland have identified both common and oncogene-specific events associated with tumor formation and progression. The tumors shared great similarities in their gene-expression profiles as compared with the normal mammary gland with an induction of cell-cycle regulators, metabolic regulators, zinc finger proteins, and protein tyrosine phosphatases, along with the suppression of some protein tyrosine kinases. Selection and hierarchical clustering of the most variant genes, however, resulted in separating the mouse models into three groups with distinct oncogene-specific patterns of gene expression. Such an identification of targets specified by particular oncogenes may facilitate development of lesion-specific therapeutics and preclinical testing. Moreover, similarities in gene expression between human breast cancers and the mouse models have been identified, thus providing an important component for the validation of transgenic mammary cancer models.

cDNA microarray | mammary cancer | oncogenes | gene-expression profiles

Gene expression profiling of human breast cancers has increased our understanding of the clinical diversity of the disease and has been instrumental in the classification of tumors into subtypes and studying their response to drug treatment (1, 2). Recently, attempts have been made to delineate pathways characteristic of hereditary breast cancer with *BRCA1* and *BRCA2* mutations (3). However, most breast cancers are sporadic, develop through the accumulation of more than one genetic lesion, and cannot be studied within the same patient, thereby posing difficulties in the identification of stage-specific events involved in the initiation and progression of cancer (4).

Many cancers in transgenic mice arise from the targeted overexpression of a particular oncogene in a well defined genetic background, thus offering particular advantages for studying tumor progression caused by a single initiating event. Although characteristic differences in the histopathology of the mammary cancers from many of these transgenic models have been defined (5), little is known about the gene-expression profiles that distinguish the tumor types on the basis of the initiating oncogenic event.

In this study, we have compared six well characterized mouse models of human breast cancer to determine the fundamental differences in gene expression between the normal mammary gland and mammary tumors and to define genes that are associated specifically with each oncogenic pathway. Gene-expression patterns of mouse mammary tumor virus long terminal repeat (MMTV)-c-*myc*, MMTV-*neu*, MMTV-*Ha-ras*, MMTV-polyoma middle T antigen (*PyMT*), C3 (1)/simian virus 40 (SV40) T/t antigen, and WAP-SV40 T/t antigen (T-ag; refs. 6–11) transgenic mice were established by high-density cDNA microarray analysis. Several of these models are directly relevant to molecular alterations found in human breast cancer. For example, amplification of *erbb2/her2/neu*, an epidermal growth factor receptor family tyrosine kinase, and the proto-oncogene, c-*myc*, have been associated with 30% and 17% of human breast cancers, respectively (12, 13). The SV40 T antigen functionally inactivates both tumor suppressor genes pRb and p53, which is often mutated in human breast cancer (14–15).

Analysis of over 8,600 unique genes demonstrated that despite different initiating oncogenic events, the mouse models were remarkably similar in their molecular expression profiles, and they differed greatly from normal mammary gland. Changes in gene expression observed in human breast cancer were often found in the mouse models. In addition, our analysis identified a small subset of genes capable of assigning oncogenic signatures to the tumor types, which may potentially be useful in identifying previously uncharacterized, oncogene-specific therapeutic targets for the treatment of human breast cancer. We propose that gene-expression profiles of mouse models of cancer will further identify models most suitable for preclinical testing of novel therapeutic molecules and serve as an important means of model validation.

Methods

cDNA Clones. The mouse oncochip of National Cancer Institute 2.7K array (2,700 features) and mouse Incyte GEM1 8.7K array (8,700 features) of cDNA clones were arrayed separately at the National Cancer Institute Applied Technology Center. The gene list is available at http://nciarray.nci.nih.gov. Approximately 11,000 features comprising 8,680 unique genes were analyzed. The cDNA set spanned 4,246 named genes, 2,288 expressed sequence tags (EST), and 2,146 Riken cDNAs.

Animals. All transgenic mice studied were of the FVB strain background. Animals were housed and cared for in accordance with National Institutes of Health guidelines and palpitated for tumors twice every week. Tumors (0.6–0.8 cm) were dissected

Abbreviations: MMTV, mouse mammary tumor virus; EST, expressed sequence tag; PCNA, proliferating cell nuclear antigen.

‡‡Present address: Genome Institute of Singapore, Singapore 117604.

§§To whom reprint requests should be addressed at: Laboratory of Cell Regulation and Carcinogenesis, 41 Library Drive, Room C619, National Institutes of Health, Bethesda, MD 20892. E-mail: jegreen@nih.gov.

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out, fixed in 4% (wt/vol) paraformaldehyde for histology, and the remainder was snap frozen in liquid nitrogen. All mammary gland tumors from the same mouse were pooled, and tumors from four to six different mice were analyzed for each mouse model. Mammary glands from 10 randomly selected 11-week old FVB females at various stages of estrous cycle were pooled and used as the reference RNA for all arrays. Three batches of reference RNA were prepared.

RNA Isolation and cDNA Microarray Analysis. RNA from the normal mammary gland and tumor samples was extracted by the guanidine isothiocyanate method (16). Total RNA from each sample (20 μg) was labeled and hybridized as described (17), except that the reactions were purified by using Microcon YM-30 columns. The array slides were scanned with an Axon 4000 scanner (Axon Instruments, Foster City, CA) at a resolution of 10 μm. The reference RNA was labeled by using Cyanine 3-dUTP (Cy3), and the tumor samples were labeled with Cyanine 5-dUTP (Cy5), except for the reverse-labeling experiments.

Data Filtering and Normalization. Image analysis and the calculation of average foreground signal adjusted for local channel specific background was performed with GENEPIX software. All statistical analyses were performed with the S+ package. Spots with signal intensities in both channels less than 100 were excluded. If at least one channel had an intensity above 100, the intensity under 100 was set at 100. The average number of clones filtered from analysis was 110 for the NCI 2.7K array and 85 for the GEM1 8.7K arrays. Each array was separately globally normalized to make the median value of log2 ratio equal to zero.

Evaluation of Gene-Specific Dye Bias. Because we consistently labeled the reference normal breast epithelium with Cy3 and the tumors with Cy5, it is possible that the down-regulation detected in tumors for some genes could reflect dye bias that was not removed by the normalization process. To evaluate this possibility, we examined pairs of arrays in which the tumor sample was labeled with Cy5 and the reference with Cy3 for one array, and the labeling was reversed in the paired array. We analyzed 17 such pairs of arrays for the NCI 2.7K array and 11 such pairs containing clones from the GEM1 8.7K array. Reverse-labeled experiments were performed by using a variety of tumor samples, including prostate tumors from a related study. We computed the average difference in log2 ratio between normalized forward- and reverse-labeled experiments for each clone as an estimate of the residual dye bias for that clone. Of the clones on the NCI 2.7K array, only 7 had an average difference in log2 ratios greater in absolute value than 1 (i.e., 2-fold difference), and for the GEM1 8.7K array, the number was 19. There was no general systematic bias favoring either dye after normalization. For comparing tumor models with each other, the use of an internal reference consistently labeled in all arrays should not result in spurious claims that genes are differentially expressed among models, but may result in missing the detection of differential expression of genes that do not stain adequately with Cy5.

Sample Clustering and Multidimensional Scaling. Average linkage hierarchical clustering of samples was based on a Pearson correlation similarity metric using all available genes or genes selected by F test (see below). Multidimensional scaling is the process of representing the distances (1 minus similarities) of a group of objects in a low dimensional (i.e., three-dimensional) space (18). Multidimensional scaling analyses were performed with the same distance matrix as was used for hierarchical clustering for genes selected by F test.

F Tests and Gene Clustering. To identify genes that distinguish transgenic mouse models from each other, an F test was performed separately for each gene represented on the arrays. The F test is a generalization of the t test for more than two groups. Statistical significance levels were calculated with all available log ratios for genes in GEM1 8.7K arrays and NCI2.7K arrays. Genes showing variation between models greater than expected at the 0.001 level of statistical significance were identified by using a stringent level of significance controls for the large number of genes tested. Approximately 10 genes significant at the 0.001 level would be expected by chance, but the statistical significance level is approximate and limited by the accuracy of the normal distribution approximation. Average linkage hierarchical analysis of these genes was performed by using a Pearson correlation similarity metric to group genes based on their patterns of variation across the transgenic models. Gene clusters were selected based on a cut of the dendrogram at a correlation coefficient of 0.7. The clusters and associated image-plots were displayed with TREEVIEW software (19).

Northern and Western Blot Analysis. Total RNA (20 μg) was electrophoresed through a 1.2% agarose formaldehyde gel, and Northern blot analysis was performed by using the method of Church and Gilbert (20). Gene-specific inserts of sequence-verified cDNA clones (Incyte Pharmaceuticals, Palo Alto, CA) were labeled with radioactive [^{32}P]dCTP by using the Ready-To-Go DNA-labeling kit (Invitrogen); blots were washed by using standard protocols and exposed to Kodak XO-MAT films. For Western blots, 30 μg of protein extracts from tumors were analyzed as described (21). Anti-proliferating cell nuclear antigen (PCNA) and anti-actin antibodies (Santa Cruz Biotechnology) were used at a 1:500 dilution. The detection of antibodies was performed by an Enhanced Chemiluminescence Kit (NEN/Du Pont).

Results

We have performed microarray analysis of mouse models of human breast cancer. The data collected was analyzed to determine "cancer-related" genes by comparing the expression profiles of tumors to that of the normal mammary gland. Secondly, individual mouse models were compared with each other to define "oncogene signatures" characteristic of the initiating oncogenic event. Results are available at the National Cancer Institute Mouse Mammary Models Collective web site: http://emice.nci.nih.gov.

Cancer Genes. Genes that were commonly regulated in mouse models as compared with normal tissue were identified as those with an average $\log_2$ ratio of at least 1 or less than -1, when the average was computed across all arrays. Such analysis resulted in the selection of 627 features from the mouse GEM1 8.7K array and 276 features from the NCI 2.7K array. The relationship between the tumor types as determined by hierarchical clustering using these genes is shown in Fig. 1. The tumor models seem to be highly similar in their expression profiles as determined by the high correlation coefficients in the dendrogram. Interestingly, tumors from the same cohort of transgenic animals clustered together. In addition, we observed modulation in several genes previously implicated in human breast cancer (Table 1). The named genes were classified by using GENECARDS (http://bioinformatics.weizmann.ac.il) and by extensive review of the literature (see Table 2, which is published as supporting information on the PNAS web site, www.pnas.org). Regardless of the transgenic model, the most striking feature of the tumors was the high induction of genes in the glycolytic pathway involved in the conversion of glucose to pyruvate, including high levels of lactate dehydrogenase. Accelerated rates of metabolism were evident by increased expression of translation elongation factors

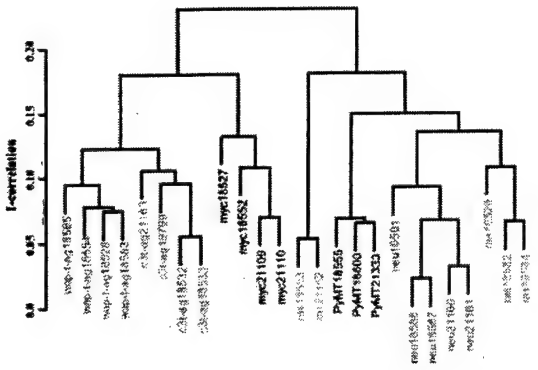

Fig. 1. Comparison of mouse models of cancer. A dendrogram depicting the degree of similarity between the six mouse models of human cancer is shown. Average linkage hierarchical clustering using Pearson correlation similarity was carried out for all genes studied with at least a two-fold geometric mean relative to the normal mammary gland (reference RNA). The scale on the right shows 1 minus correlation. The high correlation coefficients suggest that the tumors are highly similar in their gene-expression profiles, irrespective of the initiating oncogenic event.

and structural RNA genes. Several cell-cycle regulators, signaling receptors and their effectors, including G proteins and downstream transcription factors, were significantly induced in all mammary gland tumors. A unique observation of our profiling data was the induction in the expression of several zinc-finger proteins in all mouse models studied. However, expression of soluble protein tyrosine kinases was diminished in most tumors with a concomitant induction in the protein tyrosine phosphatase activity. Several cytoskeletal proteins like tubulin 4 and tubulin 5 isoforms displayed identical regulatory patterns in all tumors studied. In addition, several ESTs with as yet uncharacterized function were modulated in the tumors, thus identifying a vast number of genes potentially important in the process of oncogenesis.

Several genes were commonly down-regulated in all tumor models studied, including fat-specific gene 27, enolase, and carbonic anhydrase. It is likely that a subset of these genes appeared suppressed because of the comparison of an enriched population of epithelial cells in the tumor to a mixture of adipose and epithelial cells of the normal mouse mammary gland. We compared RNA from fat pads devoid of epithelial cells to our reference RNA to identify such a subset of genes (see Fig. 5, which is published as supporting information on the PNAS web site).

Oncogene Signatures. Analysis of commonly regulated genes was powerful in determining pathways that differed between a normal and cancerous state of mouse mammary gland but could not completely identify discrete oncogene-specific clusters that would aid the identification of pathway-specific targets for further characterization. Therefore, F tests were performed on the microarray data described in *Methods* and which resulted in the identification of a total of 930 genes. Average linked hierarchical clustering of the arrays with regard to genes identified by F tests separated the tumor types into three groups (Fig. 2*a*). The first group was composed of SV40 T/t antigen tumors. This group seemed to be more similar to the MMTV-*myc* tumors but was distantly related to the third group of tightly clustered *neu*-, *ras*-, and *PyMT*-induced tumors. Multidimensional scaling analysis of the data visually depicts the separation of the three groups (Fig. 2*b*). The image plots of the clustered genes is shown in Fig. 6 *a* and *b* (which is published as supporting information on the PNAS web site).

T-Antigen Cluster. Of the 930 genes differentially expressed between the tumor models, more than 100 genes were uniquely modulated by SV40 T/t antigen (Table 3, which is published as supporting information on the PNAS web site). This group was the largest of tumor signature genes among all of the oncogenes studied. T-ag expression perturbed several important cellular pathways, including cell cycle, DNA replication, RNA metabolism, signal transduction, cell death, and genes implicated in human cancer (Table 3). The most unique observation was the induction of calcium binding and/or calcium-regulated genes like calcyclin, calcium calmodulin-dependent kinase II (CamKII), annexin A2 and 5, caldesmon, and calumenin exclusively in the Tag group (Fig. 3, cluster c). Interestingly, only a few genes involved in DNA repair, RAB4A and pRb, showed appreciable down-regulation in these tumors.

Myc Cluster. The *myc*-derived tumors cluster closely with the T-ag tumors but display only a subset of the gene-expression changes observed in the T-ag tumors, including overexpression of *cyclin E*, *PCNA*, and *cdc25A* (Fig. 6*a*). *Myc* overexpression seemed to

Table 1. Comparison of genes that have been implicated in human breast cancer with mouse models of cancer

Gene	Status in human cancer	Status and tumor type in mouse
Cortactin	Amplification of chromosome 11q13 in 15% of breast cancers amplifies cortactin expression	Induced in *myc*
Galectin 3	Increased grade of breast tumor has decreased expression	Induced in T-ag
Tieg	Not expressed in invasive lesions but highly expressed in normal breast epithelium, moderately in noninvasive tumors	Induced expression in *neu-ras-PyMT* group
Phopholipase A2, VII	Increases in breast cancer	Induced in T-ag
Tumor antigen CO-029	High in breast carcinomas	Induced in *neu*
Aldolase C	Found in human DCIS lesions	Induced in *neu*
Prothymosin alpha	A *Myc* target used as a marker to measure proliferation index of human breast tumors	Highly induced in Tag and *myc*
STAT3	Increased activity in human breast and prostate cancer cells	Induced in models except *myc*
CD24 antigen	Differential expression in human mammary cell lines	Induced in all models
Lipocalin 2	Increased expression in normal ductal lumens surrounding cancer tissue, but low expression in normal ductal epithelium	Induced in all models
Acid β glucosidase	Inhibitors to this enzyme displayed decreased metastasis	Induced in all models
Riken cDNA	Similar to DRIM-1, down regulated in metastasis, isolated from MDA-Mb 435 cells	Induced in all models
Procollagen, Iα and IIIα	Induced in stromal cells of malignant lesions of human breast cancer	Suppressed in all tumors

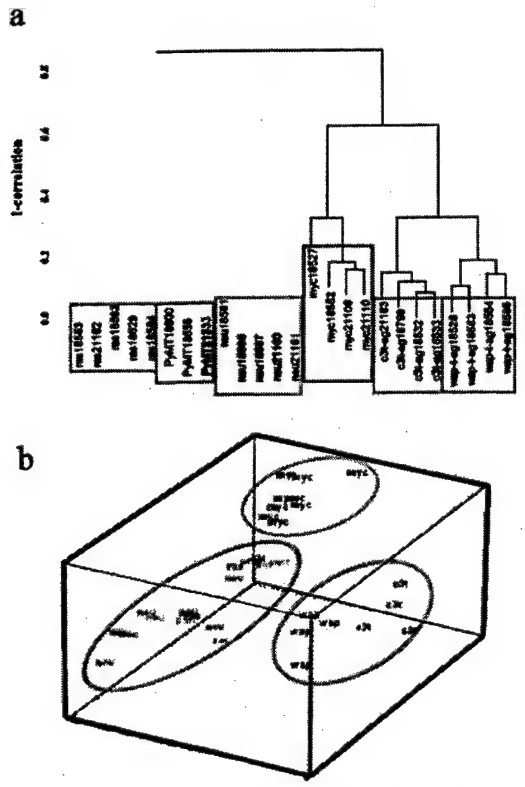

Fig. 2. Comparative cDNA microarray analysis of mouse models of human breast cancer. (*a*) A subset of 930 genes selected by F test ($P < 0.001$) were clustered as described in *Methods* to determine oncogene-specific signatures. The tumors fall into three distinct groups: (*i*) MMTV-*neu*, MMTV-*ras*, and MMTV-*PyMT*; (*ii*) MMTV-*myc*; and (*iii*) the *T antigen* group. (*b*) Data are represented in three dimensions by multidimensional scaling.

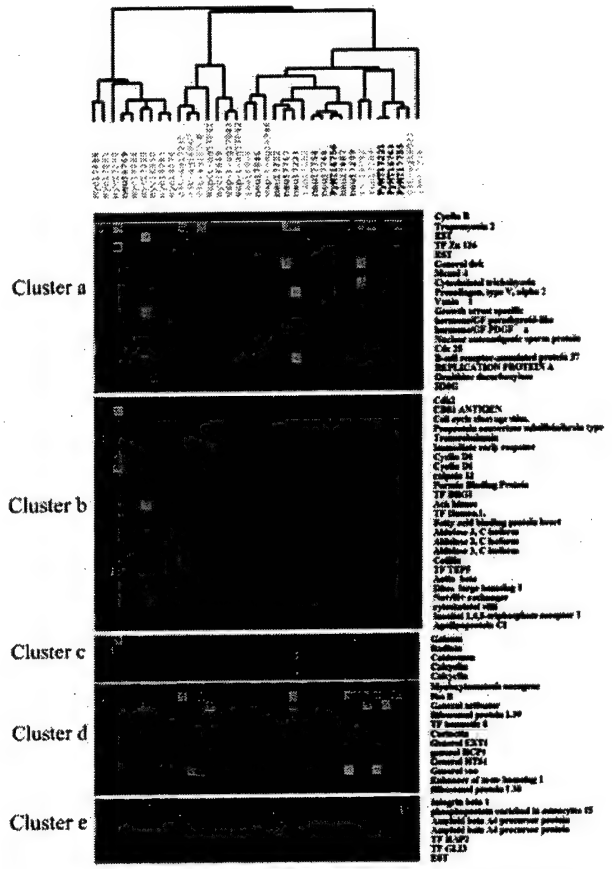

Fig. 3. Oncogene-specific clusters spanning interesting variant genes are shown. Each tumor type is color-coded; gene names are shown on the right-hand side of the figure. Complete hierarchical clustering of the 258 genes and 672 genes that varied significantly by F test ($P < 0.001$) in their expression profiles across the tumors studied are shown in Fig. 6 *a* and *b*, respectively.

suppress *Rb*- and *E2F*-related gene. In addition, the *myc* cluster included previously identified *myc* targets like *c-fos*, ornithine decarboxylase, and *dihydrofolate reductase* (22), along with up-regulation of several transcription factors like *enhancer of zeste homolog*, hox proteins, general transcription activators and repressors like *sno* and other DNA-binding proteins. As reported (23), we observed increased expression of ribosomal RNA genes in *myc* tumors.

***Neu-ras-PyMT* Cluster.** These tumors showed very similar profiles of gene expression and clustered very tightly with each other, with maximal changes observed in tumors derived from *neu* overexpression. GTPase activating proteins (GAPs) and related G proteins were predominantly induced in this group (Fig. 6*b*). In addition, *neu* tumors showed up-regulation of *E2F*, *cdk-2*, and *cyclin D1*, as has been documented earlier (24). A unique class of tetraspanin family membrane glycoproteins previously isolated as tumor antigens, including CD81 and CO-029, were up-regulated in *neu* tumors. In addition, *neu*-derived tumors displayed induction of proteases like calpains and *MMP15* as well as *extracellular proteinase inhibitor*. Within the genes analyzed, we failed to detect changes in the *PyMT* tumors that may suitably explain the high incidence of metastasis observed in this mouse model.

Validation of the Observed Gene-Expression Profiles. Northern blot analyses were performed for 11 differentially expressed genes identified by microarray studies. Of these, three known genes displayed results similar to the array data. Of the eight ESTs studied, five probes labeled poorly and could not give sufficient and/or specific signal, suggesting that EST data from arrays require rigorous validation before interpretation. Representative blots are shown in Fig. 4. The bar charts in each panel represent the levels of gene expression observed by microarray hybridization; Northern blots for the corresponding EST or named gene(s) are shown below. The N lane denotes total RNA from normal mammary glands. EST-1 was highly induced in all tumors and was recently annotated as a structural ribosomal RNA gene (Fig. 4*a*). Fig. 4*b* shows a previously uncharacterized EST (*EST-2*) that was specifically induced in the *neu* and *ras* tumors. *PCNA* was specifically induced in the *myc*-T-ag group, but this induction was absent from the *neu-ras-PyMT* group. This variant expression pattern was retained at the protein level (Fig. 4*c*). Also identified in the *myc* tumors was the specific down-regulation of *hey-1*, *hairy enhancer-of-split-related gene with YRPW motif*, involved in the Notch-signaling pathway (Fig. 4*d*). These results demonstrate that the modulations observed by cDNA microarray analyses could be validated by a second technique.

Discussion

We hypothesized that comparative gene-expression profiling of oncogene-derived tumors would broaden our understanding of

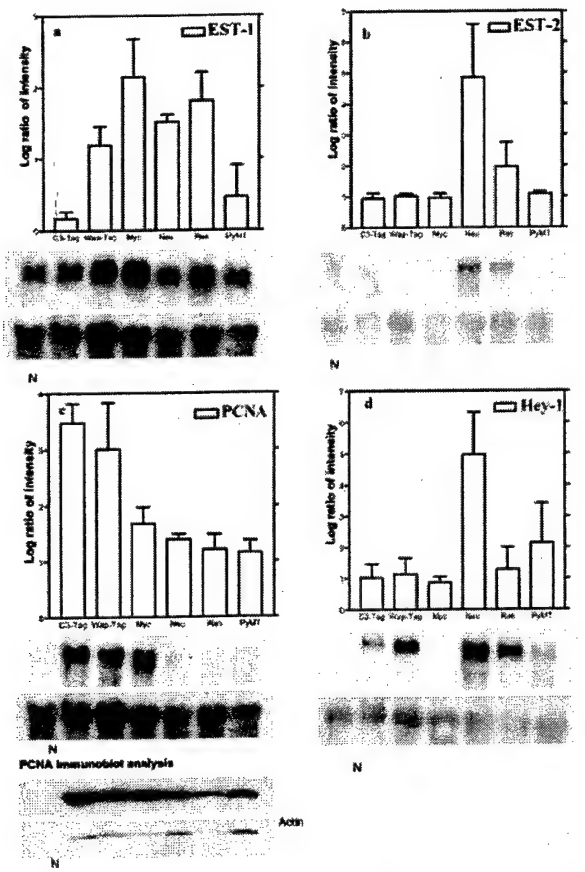

Fig. 4. Comparison of microarray and Northern blot analysis. The average log ratio of intensity (to the base 2) observed by microarray hybridization for each selected gene is depicted as a bar graph and is plotted on the *y* axis, with the tumor type on the *x* axis. (Bars = SD.) The results of Northern blot analysis performed for the gene are shown below each bar graph. Tumor samples are listed on the top of each lane of the Northern blot; the N lane denotes normal mammary gland RNA sample. (*a*) *EST-1*. (*b*) *EST-2*. *PCNA* and *Hey-1* were modulated in an oncogene-specific manner (*c* and *d*, respectively). The differential regulation of *PCNA* was retained at the protein level by Western blot. 18S rRNA was used as an internal control for RNA quantification for Northern blots.

oncogene-specific pathways, a study difficult to perform in the human disease owing to the potential multiplicity of genetic changes associated with human breast cancer. First, we determined differences in gene profiles of the normal mammary gland and mammary tumors, irrespective of the initiating oncogene, thus identifying pathways that change during tumor formation and disease progression. Second, by an analysis of variance, we studied the genes that differed in their expression profiles depending upon the initiating oncogenic event. We proposed that the first analysis would identify genes that display altered expression in mammary cancer irrespective of the causal genetic lesion. The second approach would identify genes and potential pathways uniquely perturbed by the specific initiating oncogenic event.

Results of the first analysis indicate that the mouse models studied display common and highly similar patterns of gene expression that differ greatly from the normal mammary gland. We found many similarities in the expression of genes previously implicated in human breast cancer, thus supporting the use of transgenic mouse models in studying aspects of human cancer. All mouse models displayed increased expression of transcription factors bearing the zinc finger motif and certain cytoskeletal proteins like tubulin 4 and tubulin 5. Paclitaxel, a cytotoxic agent and potent inducer of tubulin polymerization, has been used in combinatorial chemotherapy of advanced or metastatic breast cancer (25). Our data suggest that such compounds potentially could be tested *in vivo* in mouse models of cancer. Although several receptor tyrosine kinases have been targeted for breast cancer therapy, we observed suppression of several protein tyrosine kinases concomitant with the induction of protein tyrosine phosphatases, thereby identifying a unique subset of potential targets that are cancer-related but are independent of initial events in tumor formation.

We performed F tests to identify a subset of genes that constituted the oncogene-induced signatures. Hierarchical clustering with this subset of genes segregated the tumors into three major groups: the nuclear oncogene groups, (*i*) MMTV-*myc* and (*ii*) C3(1)-*T-ag* and WAP-*T-ag*, and the extranuclear signaling oncogene group, (*iii*) MMTV-*neu*, MMTV-*ras*, and MMTV-*PyMT*. The tight association of *neu-ras-PyMT* seems to be because of the convergence of the *neu*, *PyMT*, and *ras* pathways, as documented earlier (26). Despite the use of two different promoters to express T-ag [C3 (1) prostatein and WAP] with some distinct histopatholic differences in tumors, the T-ag tumors clustered tightly, suggesting that the oncogene was the primary determinant of gene-expression profiles.

T-ag inactivates both p53 and pRb, deregulating two important checkpoints in cell cycle. p53 seems to regulate gene expression at both the G_1/S transition, by induction of cell-cycle inhibitors, and G_2/M transition, by suppression of genes, including cdc2 and cdc25A (27). pRb interacts with E2F, HDAC, and Mcmd7, as well as SWI-SNF proteins that collectively result in suppression of cell-cycle genes leading to cell-cycle arrest (28). We observed induction of cdc2, cdc25A, G_1 phase cyclin E, PCNA, and histone acetylases, as well as proteins involved in DNA replication, including the Mcmd family, thereby indicating that T-ag expression results in unchecked progression through both cell-cycle check points leading to increased cell duplication. The MMTV-*myc* tumors displayed cell-cycle gene-expression profiles that overlapped with the T-ag models. In contrast, the *Neu-ras-PyMT* group was characterized by the induction of *cyclin D1*, *cdk-2*, and *E2F*, with no apparent changes in genes involved in G_2/M transition or those affecting DNA replication. This observation implies that tumors derived from *Neu-ras-PyMT* display events more similar to those that occur after mitogenic stimulation of cells and the activation of the *ras* pathway (29).

The most unique gene cluster specific to T-ag was the induction of calcium-signaling pathways and *S100A4* and *S100A1*, which have been associated with invasive lesions in mammary models of cancer (30). Treatment of breast cancer by the antiestrogen, tamoxifen, has been clinically most successful for estrogen-dependent tumors. In addition to functionally blocking estrogen receptor (ER) signaling, tamoxifen has been shown to inhibit other enzymatic activities in the cell, like those of protein kinase C and calmodulin-dependent cAMP phosphodiesterases (31). For this reason, calmodulin inhibitors are being evaluated *in vitro* as potential antiproliferative and chemopreventive agents in ER-positive as well as ER-negative human breast cancer cells (32, 33). C3(1)-T-ag tumors lose ER expression during tumor progression from mammary intra-epithelial neoplasia lesions to adenocarcinomas (21). Because only the T-ag models show an increase in calcium-signaling molecules, they may be well suited for the *in vivo* evaluation of novel calmodulin inhibitors.

Most of the genes that were modulated in *myc* tumors could be classified into three major groups: (*i*) the cell-cycle pathway, (*ii*) transcription factors, and (*iii*) ribosomal RNA genes. Interestingly, the *enhancer of zeste homolog 1* (*ezh1*) and *hoxb8* gene have been mapped to mouse chromosomes 11 (34). Increased

copy number and rearrangements of chromosome 11 have been described previously in the MMTV-*myc*-derived cell lines (35). Hence, the observed *myc*-specific induction of these genes may be a result of chromosomal aberrations in addition to increased gene expression. In contrast, the complete suppression of *hey-1*, a downstream effector of Notch signaling (36), was characteristic of most *myc* tumors analyzed, whereas it was induced in other models (Fig. 4*d*). It is possible that Notch signaling through hey-1 is specifically down-regulated in *myc*-derived tumors, because *myc* is known to transcriptionally repress several genes with diverse functions including CDK1, p21, H-ferritin, collagen, and fibronectin (37).

Tumors from transgenic mice overexpressing *neu*, *ras*, and *PyMT* clustered very tightly owing to remarkable similarities in their gene-expression profiles. However, only three changes were unique to *ras*-derived tumors, and nine were specific to *PyMT*. Many GAPs and serine-threonine kinases were up-regulated in these tumors. Increased processing of pre-pro-ligands of the epidermal growth factor super-family by induction of specific proteases belonging to the calpain and metalloprotease family has been documented previously in human cell lines and tumors bearing increased *erbB2/neu* expression (38). We observed the induction of calpains and *MMP15* mostly in *neu* tumors and in some *ras* tumors. In addition, members of the tetraspanin family including CD9, CD81, and several ESTs similar to this family were up-regulated in MMTV-*neu* tumors. However, loss of *CD9* expression is associated with invasive lesions in human breast carcinomas and is shown to be a marker of poor prognosis of the disease (39, 40).

Gene-based approaches have been successful in breast cancer therapy and have led to the development of several classes of drugs (41). Often, combinatorial drug therapy is more effective for the treatment of advanced cancer than single agents, where development of drug resistance is common. Microarray technology offers a high throughput screening to help identify such targets that may lead to "smart" combinatorial therapies for the treatment of clinically diverse human disease. We have profiled late-stage tumors from six mouse models of human breast cancer, thus developing a comparative platform for cancer-related genes and/or oncogene-specific pathways. However, cross-species differences may exist in gene-expression patterns, gene functions in the process of oncogenesis, and in the metabolism of the selected candidate therapeutic agent(s). Moreover, certain changes may be limited to the specific genetic background of the mouse strain used in the present study. After careful consideration of these issues, our database may be used to select a mouse model for particular purposes, including preclinical testing of potential targets depending upon the status of that pathway in the model. Moreover, comparative expression profiling of tumors at various stages of development in mouse models would profoundly enhance our knowledge of progressive genetic changes associated with oncogene-induced events.

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1. Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., *et al.* (2000) *Nature (London)* **406,** 747–752.
2. Perou, C. M., Jeffrey, S. S., van de Rijn, M., Rees, C. A., Eisen, M. B., Ross, D. T., Pergamenschikov, A., Williams, C. F., Zhu, S. X., Lee, J. C., *et al.* (1999) *Proc. Natl. Acad. Sci. USA.* **96,** 9212–9217.
3. Hedenfalk, I., Duggan, D., Chen, Y., Radmacher, M., Bittner, M., Simon, R., Meltzer, P., Gusterson, B., Esteller, M., Kallioniemi, O. P., *et al.* (2001) *N. Engl. J. Med.* **344,** 539–548.
4. Bieche, I. & Lidereau, R. (1995) *Genes Chromosomes Cancer* **4,** 227–251.
5. Cardiff, R. D., Anver, M. R., Gusterson, B. A., Hennighausen, L., Jensen, R. A., Merino, M. J., Rehm, S., Russo, J., Tavassoli, F. A., Wakefield, L. M., *et al.* (2000) *Oncogene* **19,** 968–988.
6. Leder, A., Pattengale, P. K., Kuo, A., Stewart, T. A. & Leder, P. (1986) *Cell* **45,** 485–495.
7. Bouchard, L., Lamarre, L., Tremblay, P. J. & Jolicoeur, P. (1989) *Cell* **57,** 931–936.
8. Tremblay, P. J., Pothier, F., Hoang, T., Tremblay, G., Brownstein, S., Liszauer, A. & Jolicoeur, P. (1989) *Mol. Cell. Biol.* **9,** 854–859.
9. Guy, C. T., Cardiff, R. D. & Muller, W. J. (1992) *Mol. Cell. Biol.* **12,** 954–961.
10. Tzeng, Y. J., Guhl, E., Graessmann, M. & Graessmann, A. (1993) *Oncogene* **8,** 1965–1971.
11. Maroulakou, I. G., Anver, M., Garrett, L. & Green, J. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11236–11240.
12. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A. & McGuire, W. L. (1987) *Science* **235,** 177–182.
13. Wright, C., Angus, B., Nicholson, S., Sainsbury, J. R., Cairns, J., Gullick, W. J., Kelly, P., Harris, A. L. & Horne, C. H. (1989) *Cancer Res.* **49,** 2087–2090.
14. Mietz, J. A., Unger, T., Huibregtse, J. M. & Howley, P. M. (1992) *EMBO J.* **11,** 5013–5020.
15. DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E. & Livingston, D. M. (1988) *Cell* **54,** 275–283.
16. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159.
17. Hegde, P., Qi, R., Abernathy, R., Gay, C., Dharap, S., Gaspard, R., Earle-Hughes, J., Snesrud, E., Lee, N. H. & Quackenbush, J. (2000) *BioTechniques* **29,** 548–562.
18. Khan, J., Simon, R., Bittner, M., Chen, Y., Leighton, S. B., Pohida, T., Smith, P. D., Jiang, Y., Gooden, G. C., Trent, J. M. & Meltzer, P. S. (1998) *Cancer Res.* **58,** 5009–5013.
19. Eisen, M. B., Spellman, P. T., Brown, P. O. & Botstein, D. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14863–14868.
20. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci USA* **81,** 1991–1995.
21. Yoshidome, K., Shibata, M. A., Couldrey, C., Korach, K. S. & Green, J. E. (2000) *Cancer Res.* **60,** 6901–6910.
22. Coller, H. A., Grandori, C., Tamayo, P., Colbert, T., Lander, E. S., Eisenman, R. N. & Golub, T. R. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 3260–3265.
23. Guo, Q. M., Malek, R. L., Kim, S., Chiao, C., He, M., Ruffy, M., Sanka, K., Lee, N. H., Dang, C. V. & Liu, E. T. (2000) *Cancer Res.* **60,** 5922–5928.
24. Lee, R. J., Albanese, C., Fu, M., D'Amico, M., Lin, B., Watanabe, G., Haines, G. K., III, Siegel, P. M., Hung, M. C., Yarden, Y., *et al.* (2000) *Mol. Cell. Biol.* **20,** 672–683.
25. Esteva, F. J., Valero, V., Pusztai, L., Boehnke-Michaud, L., Buzdar, A. U. & Hortobagyi, G. N. (2001) *Oncologist* **6,** 133–146.
26. Dankort, D. L. & Muller, W. J. (2000) *Oncogene* **19,** 1038–1044.
27. Taylor, W. R. & Stark, G. R. (2001) *Oncogene* **20,** 1803–1815.
28. Harbour, J. W. & Dean, D. C. (2000) *Genes Dev.* **14,** 2393–2409.
29. Frame, S. & Balmain, A. (2000) *Curr. Opin. Genet. Dev.* **10,** 106–113.
30. Wang, G., Rudland, P. S., White, M. R. & Barraclough, R. (2000) *J. Biol. Chem.* **275,** 11141–11146.
31. Colletta, A. A., Benson, J. R. & Baum, M. (1994) *Breast Cancer Res. Treat.* **31,** 5–9.
32. Newton, C. J., Eycott, K., Green, V. & Atkin, S. L. (2000) *J. Steroid Biochem. Mol. Biol.* **73,** 29–38.
33. Jacobs, E., Bulpitt, P. C., Coutts, I. G. & Robertson, J. F. (2000) *Anticancer Drugs* **11,** 63–68.
34. Laible, G., Haynes, A. R., Lebersorger, A., O'Carroll, D., Mattei, M. G., Denny, P., Brown, S. D. & Jenuwein, T. (1999) *Mamm. Genome* **10,** 311–314.
35. Weaver, Z. A., McCormack, S. J., Liyanage, M., du Manoir, S., Coleman, A., Schrock, E., Dickson, R. B. & Ried, T. (1999) *Genes Chromosomes Cancer* **25,** 251–260.
36. Leimeister, C., Externbrink, A., Klamt, B. & Gessler, M. (1999) *Mech. Dev.* **85,** 173–177.
37. Grandori, C., Cowley, S. M., James, L. P. & Eisenman, R. N. (2000) *Annu. Rev. Cell Dev. Biol.* **16,** 653–699.
38. Yarden, Y. & Sliwkowski, M. X. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 127–137.
39. Shi, W., Fan, H., Shum, L. & Derynck, R. (2000) *J. Cell Biol.* **148,** 591–602.
40. Berditchevski, F. & Odintsova, E. (1999) *J. Cell Biol.* **146,** 477–492.
41. Bange, J., Zwick, E. & Ullrich, A. (2001) *Nat. Med.* **7,** 548–552.

APPENDIX 2

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Epidermal growth factor inhibition of c-Myc-mediated apoptosis through Akt and Erk involves Bcl-x_L upregulation in mammary epithelial cells

Danica Ramljak,[a,*] Christine M. Coticchia,[a] Tagvor G. Nishanian,[a] Motoyasu Saji,[b] Matthew D. Ringel,[b] Suzanne D. Conzen,[c] and Robert B. Dickson[a]

[a] *Department of Oncology, Lombardi Cancer Center, Georgetown University, Washington, DC 20057, USA*
[b] *Section of Endocrinology, Medstar Research Institute, Washington Hospital Center, Washington, DC 20010, USA*
[c] *Department of Medicine, Section of Hematology/Oncology, University of Chicago, Chicago, IL 60637, USA*

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Abstract

In earlier studies, we and others have established that activation of EGFR can promote survival in association with upregulation of Bcl-x_L. However, the mechanism responsible for upregulation of Bcl-x_L is unknown. For the current studies we have chosen pro-apoptotic, c-Myc-overexpressing murine mammary epithelial cells (MMECs) derived from MMTV-c-Myc transgenic mouse tumors. We now demonstrate that EGFR activation promotes survival through Akt and Erk1/2. Blockade of EGFR kinase activity and the PI3-K/Akt and MEK/Erk pathways with pharmacological inhibitors resulted in a significant induction of cellular apoptosis, paralleled by a downregulation of both Akt and Erk1/2 proteins. Consistent with a survival-promoting role of Akt, we observed that constitutively activated Akt (Myr-Akt) inhibited apoptosis of pro-apoptotic, c-Myc-overexpressing cells following the inhibition of EGFR tyrosine kinase activity. In addressing possible downstream effectors of EGFR through activated Akt, we detected significant upregulation of Bcl-x_L protein, suggesting this pro-survival protein is a target of Akt in MMECs. By using pharmacological inhibitors of PI3-K/Akt and MEK/Erk together with dominant-negative Akt and Erk1 we observed the decrease in Bcl-x_L protein. Our findings may be of importance for understanding the emerging role of Bcl-x_L as a potential marker of poor prognosis in breast cancer.

Keywords: Akt; Erk; Bcl-x_L; Epidermal growth factor receptor; c-Myc

Introduction

The c-*myc* gene is thought to play an important role in the onset and progression of breast cancer, where it is commonly amplified and/or overexpressed [1,2]. Depending on the availability of survival factors, cells that constitutively express c-Myc undergo proliferation, growth arrest, or apoptosis through poorly defined mechanisms. Previously, we demonstrated that an epidermal growth factor receptor (EGFR)-mediated survival signaling pathway(s) inhibited apoptosis in c-Myc-induced transgenic mouse mammary tumors [3]. A comparison of tumors and cell lines derived from bitransgenic *tgf a/c-myc* mice with those from single transgenic c-*myc* mice indicated that only the latter model contained a significant fraction of apoptotic cells [3], suggesting that transforming growth factor α (TGFα) protects c-Myc-overexpressing cells from apoptosis in vivo. Further in vitro studies of c-Myc-overexpressing mammary tumor-derived cells confirmed that EGFR ligands, acting through EGFR tyrosine kinase activity, suppressed apoptosis and upregulated the survival molecule Bcl-x_L, at both mRNA and protein levels [4]. However, it was not clear which pathways downstream of EGFR are responsible for these effects.

Presently, there is limited information on the signaling pathways linking EGFR to the regulation of cellular sur-

* Corresponding author: Research Building, Room W417, Georgetown University, 3970 Reservoir Road, NW, Washington, DC 20057, USA. Fax: +1-202-687-7505.
E-mail address: ramljakd@georgetown.edu (D. Ramljak).

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vival in mouse and human mammary epithelial and carcinoma cells. However, studies in some nonmammary epithelial cells (hepatic carcinoma cells and keratinocytes) have identified two survival pathways downstream of EGFR: phosphatidylinositol 3-kinase (PI3-K)/Akt and extracellular signal-regulated kinase (Erk1/2). In most cases, the PI3-K/Akt pathway delivers the most potent survival signal downstream of EGFR [5,6].

Akt is a serine–threonine kinase, downstream of PI3-K, which delivers strong survival signals in many cell types [7–9]. Both growth factors and integrins activate Akt through activation of PDK1 and putative PDK2 kinase that subsequently phosphorylate Akt at Thr308 and Ser473 respectively [10]. There are several isoforms of Akt (Akt1, Akt2, Akt3); each has been shown to be expressed at different levels in various tissues [11]. The targets of Akt in epithelial cells, including mouse and human breast cells, include proteins involved in cell growth, metabolism, and apoptosis. The Akt targets involved in apoptosis include Bad, a pro-apoptotic member of the Bcl-2 family of proteins [12], caspase 9 [13], and the forkhead transcription factor [14]. Recently, Akt also has been reported to upregulate the expression of anti-apoptotic proteins in lymphoid cells such as Bcl-x_L [15], Bcl-2 [16], and Mcl-1 [17]. Akt activates NF-κB in both fibroblasts and epithelial cells [18,19].

In most cell types, both growth factors and integrins are capable of activating the MAPK/Erk pathway. Of particular relevance to our studies, the MAPK/Erk pathway was previously shown to convey survival signals in response to EGF [20,21]. Recently, it has been shown that the PI3-K/Akt and the MAPK/Erk pathways can cooperate in the inhibition of Bad in some cell types [22]. However, the pro-survival targets and the interactions of EGFR-activated PI3K/Akt and MAPK/Erk pathways have not been established in murine mammary epithelial cells (MMECs), human breast epithelial cells, or human carcinomas. Our preliminary data indicated that Bad is expressed in MMECs; however, the phosphorylation status of endogenous Bad was difficult to determine due to the lack of reliable antibodies. With the emerging role of activated EGFR in breast cancer, we believed it would be important to determine the anti-apoptotic targets of EGFR-stimulated Akt and Erk, in MMECs and human breast cancer cells.

In our investigations to determine which survival molecules downstream of the EGFR are responsible for upregulation of Bcl-x_L and inhibition of c-Myc-mediated apoptosis in MMECs, we show that constitutively activated Akt provides protection from c-Myc-mediated apoptosis in association with upregulation of the Bcl-x_L protein. By using pharmacological inhibitors of both PI3-K/Akt and MEK/Erk1/2, and dominant-negative (DN)-Erk1 and DN-Akt we detected significant decreases in Bcl-x_L protein expression.

In conclusion, our results demonstrate that EGFR-dependent Akt activity provides a major survival signal against c-Myc-mediated apoptosis in MMECs; both Akt and Erk are obligatory for regulation of Bcl-x_L expression in this model. To our knowledge, these studies provide the first comprehensive evaluation of the role of EGFR-dependent survival molecules in inhibition of c-Myc-mediated apoptosis in murine models of breast cancer.

Experimental procedures

Cell culture and viral infection

Myc83 cells (derived from an MMTV-c-*myc* transgenic mouse mammary tumor in our laboratory) and Comma D cells (immortalized mouse mammary epithelial cells obtained from D. Medina, Baylor College of Medicine) [23] were maintained in a humidified 5% CO_2 environment, in complete medium containing: IMEM (Gibco-BRL, Gaithersburg, MD, USA), 2.5% fetal calf serum (FCS), 10 ng/ml EGF (Upstate Biotechnology Incorporated (UBI), Lake Placid, NY, USA), and 5 μg/ml insulin (Biofluids, Rockville, MD, USA). Myc83 cells were selected as a model because of their high propensity to apoptose after removal of EGF. Comma D cells (with normal c-Myc levels and mutated p53) were used to compare the potency of EGF in activating both Akt and Erk1/2 in MMECs with different c-Myc levels. Retroviruses for Myr-Akt (obtained from N. Hay, University of Illinois) [24] were made by transient transfection of retroviral pBabePuro-Myr-Akt, using Effectene Transfection Reagent (Qiagen, Valencia, CA, USA) into amphotrophic Phoenix cells (a gift of Dr. Gary Nolan, Stanford University, Palo Alto, CA, USA). Viral supernatants were collected and filter-purified. Myc83 cells were infected with pBabePuro vector only or pBabe Myr-Akt in the presence of 4 μg/ml polybrene, and infected stable clones were selected with puromycine (Sigma, St. Louis, MO, USA). Clones were pooled for further analysis. All retrovirally transduced cells were grown in complete medium, with or without addition of EGF. When testing sensitivity to apoptosis, cells were grown in IMEM only, without EGF, FCS, or insulin, or in IMEM with only EGF.

cDNA constructs and transfections

A plasmid containing dominant-negative Akt cDNA (DN-Akt in PCIS2 vector) was kindly provided by Dr. M. Kohn (NIH, Bethesda, MD, USA). The DN-Akt mutant was generated by replacing Thr308 and Ser473 with alanine. Constructs containing dominant-negative Erk1 (K71R) and empty vector pCEP4 were provided by Dr. M. Cobb (University of Texas, Southwestern Medical Center).

Transfections were performed using FuGene 6 (Roche Molecular Biochemicals). Briefly, cells were seeded in 10-cm^2 plates in complete IMEM medium and allowed to

attach overnight. Transfections were performed on 60–80% confluent cells the next day after seeding according to the manufacturer's instructions (Roche). Cell were grown 48 h posttransfections and samples were collected for immunoblotting.

Antibodies and reagents

Rabbit polyclonal anti-total Akt phospho-specific antibodies-recognizing Ser473 and Thr308; anti-Erk1 and Erk2 and anti-Erk1/Erk2 phospho-specific antibodies; Bad and phospho-Bad (Ser 112 and Ser 136) were from Cell Signaling Biolabs (Beverly, MA, USA). Anti-Akt1, anti-Akt2, and Akt3 were from Upstate Biotechnology (UBI), mouse monoclonal anti-α-tubulin and anti-Bcl-2 were from Neomarkers (Fremont, CA, USA); anti-Bcl-x_L (H-62), anti-14-3-3 (C-16), rabbit polyclonal poly (ADP-ribose) polymerase (PARP) (H-250), anti-GSK-3β(0011-A), anti-p85 PI3-K (Z-8), and anti-Raf-1(C-12) were from Santa Cruz (Santa Cruz, CA, USA). The ECL detection reagent was from Amersham (Arlington Heights, IL); PI3-K inhibitor LY 294002 (Biomol Research Laboratories, Plymouth Meeting, PA, USA) and EGFR inhibitor PD153035 (Calbiochem, San Diego, CA, USA); and MAPK kinase (MEK) inhibitor U0126 was from Cell Signaling Biolabs.

EGF stimulation and treatments with inhibitors

Myc83 and Comma D cells (1–2 $\times$ 10^6/ml) were grown in 10-cm^2 culture dishes in complete IMEM medium containing 2.5% FCS, 10 ng/ml EGF (UBI), and 5 μg/ml insulin (Biofluids) until cells reached 60–70% confluence. Complete medium was removed, cells were washed with IX PBS to remove serum and growth factors, and synchronized by serum starvation (grown in IMEM medium with 0.1% fetal bovine serum) for 24 h. After two washes with IX PBS, the cells were stimulated with IMEM medium containing 10 ng/ml EGF for 1, 2.5, 5, 10, and 20 min, washed twice with IX PBS, and lysed in buffer (Cell Signaling Biolabs) containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) for protein analysis. Insoluble material was removed by centrifugation. Protein concentrations were determined by a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) and samples were stored at −80°C until used for immunoblotting and in vitro kinase assays.

To test the requirement of active EGFR for EGF-induced Akt and Erk1/Erk2 activation, the synchronized Myc83 cells (as described above) were pretreated with 1 μM PD153035 EGFR tyrosine kinase inhibitor for 3 h, followed by a treatment with 10 ng/ml EGF at 2.5, 5, and 10 min, as described above. The concentration of PD153035 was selected based on literature and pilot studies in our laboratory (data not shown). The control Myc83 cells (−PD153035) were pretreated with an equal volume (10 μL) of DMSO and later stimulated with 10 ng/ml EGF for 2.5 and 5 min. Comma D cells were pretreated with the same concentration of PD153035 (1 μM) and for the same time (3 h) as Myc83 cells, and they were similarly stimulated with 10 ng/ml EGF for 1, 2.5, and 5 min. Control Comma D cells (−PD153035) were treated with EGF for 1, 2.5, 5, 10, and 20 min. Proteins were analyzed for the activity of both Akt and Erk1/Erk2 by immunoblotting, as described in the Western Blotting section of Experimental Procedures.

Induction of apoptosis by prolonged treatment with inhibitors of P13-K, MEK, and EGFR

Myc83pBabePuro and Myc83-Myr-Akt cells were grown in complete IMEM medium to 70–80% confluence in 10-cm^2 plates, and then the complete medium was replaced with IMEM medium containing the following: 10 ng/ml EGF (without serum and insulin); 50 μM LY294002 (Biomol) with and without the EGF; 10 μM U0126 (Cell Signaling Biolabs) with and without EGF, 1 μM PD153035 (Biomol) with and without the EGF. Cells were usually grown for 48 h, but in some cases cells were harvested after only 24 h. In one set of plates, cells were lysed for protein analysis, as described below, while another set of cells, treated equally, was used for evaluation of apoptosis by Hoechst staining.

To test if EGF rescues the Myc83 cells from apoptosis, we performed a separate set experiments in which cells were grown in complete IMEM medium to 70–80% confluence in six-well plates, and then complete medium was replaced with IMEM medium containing EGF ranging from 10 to 60 ng/ml (without serum and insulin). Cells were grown in the presence of 1 μM PD153035 (Biomol) with and without the EGF and, after 48 h were evaluated by Hoechst staining.

Measurement of apoptosis

Apoptosis was evaluated by immunoblotting of PARP protein cleavage, as described in the Western Blotting section of Experimental Procedures, and by Hoechst staining. Briefly, Hoechst staining was performed as follows: after 48 h of treatment with LY294002, U1026, and PD153035, all adherent and floating cells were collected. Samples were centrifuged for 8 min at 1000*g* at 4°C. Supernatants were discarded, and cell pellets were resuspended in IX PBS containing formaldehyde and Nonidet P-40 (NP-40) and stained with 10 μg/ml Hoechst 33258 dye for apoptotic analysis. At least 500 cells per treatment group were counted with a hemocytometer and evaluated for the presence of condensed nuclei and overall apoptotic appearance.

Akt kinase assay

Protein lysates from control and EGF-stimulated Myc83 and Comma D cells (0, 1, 2.5, 5, 10, and 20 min) were lysed in the buffer described above. Akt kinase activity was analyzed using an Akt Kinase Assay Kit (NEB), which employs GSK-3 as a substrate, according to the manufacturer's instructions. In short, 300 μg of cellular protein was immunoprecipitated with a total Akt antibody immobilized to agarose beads (NEB) at 4°C overnight. Immunoprecipitated Akt protein on beads was washed twice with 1 ml of lysis buffer (described above) and once with kinase buffer (25 mM Tris, pH 7.5; 5-mM β-glycerolphosphate, 2 mM DTT, 0.1 mM Na_3 Vo_4, 10 $MgCl_2$). The beads were then resuspended in 40:1 kinase buffer, containing the Akt protein substrate (1 μg of GSK-3 α/β fusion protein), supplemented with 200 μM of ATP. The assay was carried out according to the manufacturer's instructions (NEB), and protein samples were loaded on 12% SDS–polyacrylamide gels (Novex) and Western immunoblotting was performed, as described below. The membranes were probed with a phospho-specific antibody recognizing GSK-3α-P (at 1:1000 dilution) when phosphorylated by Akt on serine 21 (NEB). The secondary antibody used at 1:2000 was a rabbit polyclonal (NEB). Blots were developed using ECL reagents (Amersham) and exposed to ECL film (Amersham). The intensity of the bands was quantified using a Chemilmager 5500 (Alpha Innotech Corp., San Leandro, CA, USA).

Western blotting

Cell lysates containing a total of 10–15 μg of protein were used for the Western blot analysis. A PhosphoPlus MAPK antibody kit was used according to the manufacturer's recommendations (Cell Signaling Biolabs), to determine Erk activation by immunoblotting in all experimental and control cells. Cellular extracts were analyzed with antibodies against total Erk1/2 (p44 and p42) and with phospho-specific antibodies against phospho-p44/42 (Thr202/Tyr204) (Cell Signaling Biolabs). In addition to Akt kinase assays (see above) the total activity of Akt was determined using phospho-specific antibodies recognizing Akt specifically phosphorylated at Ser^{473} or Thr^{308} (Cell Signaling Biolabs). Akt protein expression was determined by using an antibody recognizing total Akt independent of phosphorylation status (Cell Signaling Biolabs).

All samples for Akt, Erk1/2, p85 PI3-K, Raf-1, and PARP were analyzed on 8% SDS–polyacrylamide gels (Invitrogen, Carlsbad, CA, USA). Those probed for GSK-3β used 10% SDS–polyacrylamide gels, while samples analyzed for Bcl-2, 14-3-3, and Bcl-x_L were tested on 12% SDS–polyacrylamide gels (Novex). Proteins were transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA, USA) by electroblotting. After transfer, membranes were stained with Ponceau protein stain (Sigma) to test for equal loading. The membranes were then washed with IX phosphate-buffered saline /0.1% Tween 20 (PBST) 3 × 10 min. After washing, membranes were blocked with 2% bovine serum albumin (BSA) in for 1 h. After incubation in primary antibody overnight at 4°C, all blots were washed for 3 × 10 min with PBST and probed for 1 h with the corresponding secondary antibody (anti-mouse or anti-rabbit labeled with horseradish peroxidase; from Amersham, Arlington Heights, IL, USA, or from NEB). After three repeated washes in PBST the blots were developed using a chemiluminescence ECL kit (Amersham) and exposed to X-ray film (Amersham). On several occasions, to test equality of loading, membranes were stripped and reprobed with an antibody recognizing α-tubulin, a 57-kDa protein (Neomarkers). The results of immunoblotting were quantified by densitometry using Chemilmager (Alpha Innotech Corp., San Leandro, CA, USA) and the significance of differences in band intensity was evaluated with a Student's t test.

Results

EGF is a potent activator of Akt in mouse mammary epithelial cells

Our previous studies showed that activation of the EGFR by either EGF or TGFα delivers a potent survival signal to mouse mammary epithelial cells overexpressing c-Myc, both in vivo and in vitro [3]. However, it was not clear which survival pathways downstream of the EGFR were responsible for the inhibition of apoptosis. In the present work, we show that in the absence of serum, EGF stimulation of both Myc83 (MMECs derived from mouse transgenic for c-Myc) and Comma D (immortalized MMECs) significantly activates Akt, a potent survival molecule in fibroblasts and several other epithelial cell types [8]. Akt is activated by 10 ng/ml EGF, within 1 min, (2-fold increase), in both Myc83 (Fig. 1A) and Comma D cells (Fig. 1C). Maximum Akt activation in Myc 83 cells (4-fold increase) was seen at 5 min, followed by a decrease to basal activity by 10 min (Fig. 1A). Similar results were obtained for Comma D cells (Fig. 1D). EGF activation of Akt resulted in the phosphorylation of Ser473 (Figs. 1A and D) and Thr308 (data not shown). Phosphorylation of both sites is required for full activation of Akt [9]. As expected, the EGF treatment did not affect the expression levels of total Akt protein in either cell line, as shown by immunoblotting using anti-Akt antibody recognizing all three Akt isoforms (Figs. 1B and E). The activity of Akt, following EGF stimulation, was confirmed by an Akt kinase assay, using GSK-3α fusion protein as a substrate (Figs. 1C and F). Akt has been shown to phosphorylate GSK-3α at serine 21 [25]. By using an antibody that specifically recognizes

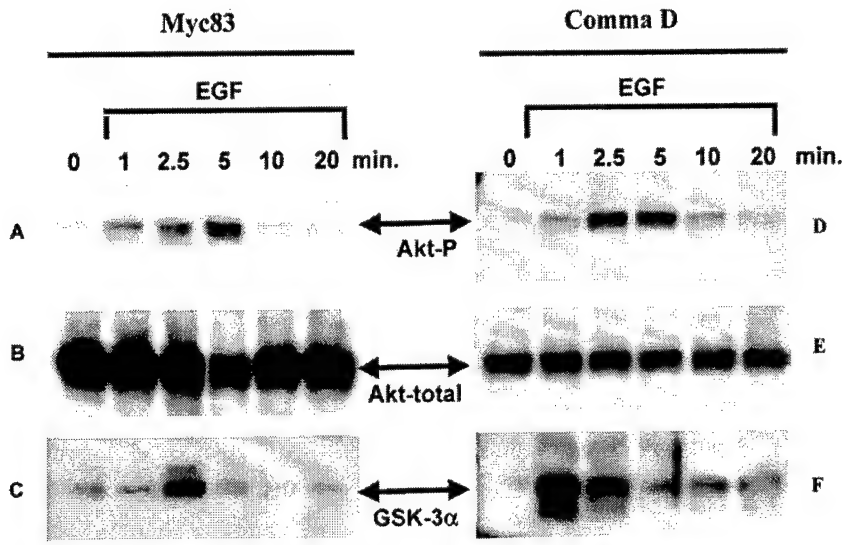

Fig. 1. EGF is a strong activator of Akt in MMECs. (A) Myc83 cells were synchronized by serum starvation (in 0.1% FCS) for 24 h and stimulated with 10 ng/ml EGF in the absence of serum for 1, 2.5, 5, 10, and 20 min. The cells were lysed, and activation of Akt was assayed by immunoblotting, using a phospho-specific antibody recognizing Akt when phosphorylated (activated) at Ser 473. (B) Phospho-Akt blots were stripped and reprobed with an Akt antibody that recognizes Akt independent of its phosphorylation status. (C) In addition, the phosphorylation status of Akt was analyzed by an Akt kinase assay using GSK-3α as a substrate in vitro (D, E). (D–F) Similarly, the effects of EGF on Akt activation and expression also were evaluated in non-c-Myc-overexpressing Comma D cells by immunoblotting and kinase assay. These results are representative of three identical, independent assays using the same lysates and two identical assays using different lysates.

phospho-GSK-3α at serine 21, we confirmed the phosphorylation of GSK-3, a known target of activated Akt, in both Myc83 (Fig. 1C) and Comma D (Fig. 1F). Interestingly, in Comma D cells the maximal activation of GSK-3α at serine 21, as detected by Akt kinase assay, preceded the activation of Akt as detected by immunoblotting. The mechanism responsible is not clear, but it is possible that activation of some other EGFR-activated kinase might precede the activation of Akt and cause phosphorylation of GSK-3α in Comma D cells.

To determine which of the three major Akt isoforms (Akt1, Akt3, Akt3) are expressed and activated after EGF treatment in MMECs, we evaluated their expression levels in Myc83 and Comma D by using antibodies specifically recognizing each isoform (UBI). We detected significant expression of Akt1 and Akt2 in all MMECs analyzed (data not shown), while expression of Akt3 could not be detected in any of the cells with the antibody used in this study (data not shown).

Activation of Erk1/Erk2 by EGF parallels Akt activation

Although in the majority of cell lines tested to date, the PI3-K/Akt pathway has been shown to deliver a stronger

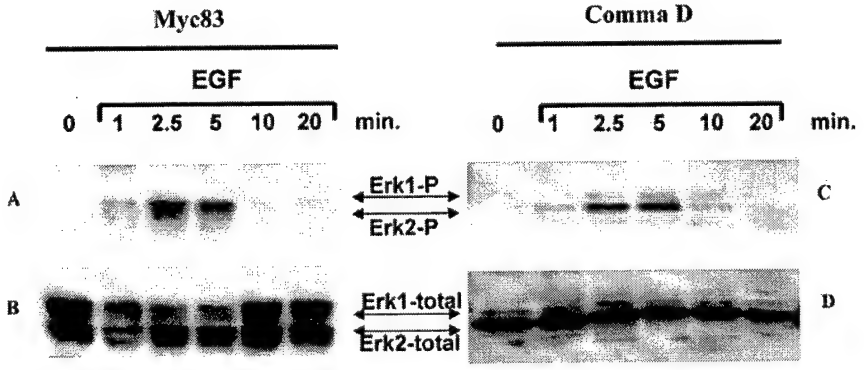

Fig. 2. EGF is a potent activator of Erk1/2 in MMECs. EGF at 10 ng/ml strongly activated Erk1/2 within a time frame similar to that shown for Akt, in both Myc83 (A) and Comma D (C) cells. Phosphorylation status of Erk1/2 was determined by immunoblotting using antibody (NEB) recognizing phospho-Erk1/2. Both blots for phospho-Erk were stripped and reprobed with antibody recognizing Erk1/2 independent of phosphorylation status in both Myc83 (B) and Comma D (D) cells.

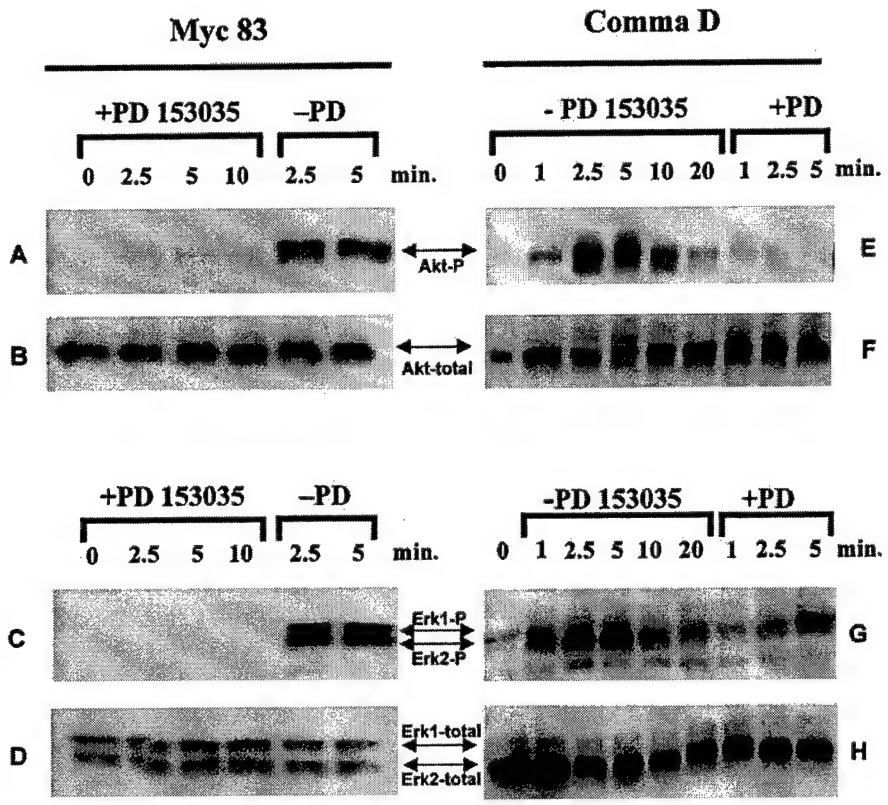

Fig. 3. Effect of a pharmacological inhibitor of EGFR kinase activity on activation and expression of both Akt and Erk1/2 in MMECs. The EGFR tyrosine kinase inhibitor PD153035 (1 μM) completely blocked activity of Akt in Myc83 (A) and Comma D (E) cells. The blots were reprobed with an antibody recognizing total Akt to demonstrate that while PD153035 inhibits the activation of Akt, it has no effect on Akt expression in Myc83 (B) and in Comma D (F) cells. While treatment with PD153035 completely blocked the activation of Erk1/2 in Myc83 cells (C), it did not affect the activation of Erk1/2 in Comma D cells (G). Blots for phospho Erk1/2 were reprobed with antibody recognizing total Erk1/2. Expression of Erk1/2 was unchanged by PD153035 treatment in both Myc83 (D) and Comma D (H) cells. These results are representative of two independent assays.

survival signal compared to the MEK/Erk pathway, in some epithelial cells, both pathways appear to be equally important in delivering survival signals [26]. Therefore, we next determined the status of Erk1/Erk2. The results show that EGF is capable of specifically activating Erk1/Erk2 in both cell lines (Figs. 2A and C), within a similar time frame as shown for Akt (Figs. 1A and D). In each cell line, the highest Erk1/Erk2 activity was detected at 2.5 and 5 min (2.5-fold); similar to Akt, the activity declined by 10 min. In Myc83 cells, the signal for Erk1 was the stronger (3-fold increase) of the two isoforms (Fig. 2A), while in Comma D cells, Erk2 was predominantly activated (2.5-fold) (Fig. 2C). The expression of total protein levels for Erk1 and Erk2 (Figs. 2B and D) was mostly unchanged by EGF treatment in both cells, with the exception of samples with phosporylated Erk1/2 in Myc83 cells, in which we detected less of total Erk1/2. Although the mechanism responsible is not clear, it is possible that the variation in Erk immunoreactivity, shown in Fig. 2, may simply reflect differential reactivity of the phosphorylated species of these kinases, rather than regulation of their levels.

Inhibition of EGFR tyrosine kinase prevents activation of both Akt and Erk1/Erk2 in c-Myc-overexpressing MMECs

To test the requirement of activated EGFR kinase in the activation of Akt, Erk1, and Erk2, we blocked EGFR tyrosine kinase with PD153035, followed by EGF treatment. In both Myc83 and Comma D cells, inhibition of EGFR kinase activity completely abolished EGFR-mediated activation of Akt (Figs. 3A and E), without affecting total protein levels (Figs. 3B and F). As expected, EGF treatment strongly activated Akt in cells treated with vehicle (DMSO) (Figs. 3A and E). However, only in Myc83 cells was blockade of the EGFR tyrosine kinase activity capable of inhibiting activation of Erk1/Erk2 (Fig. 3C), without affecting the expression levels of Erk1/Erk2 proteins (Fig. 3D). Interestingly, inhibiting EGFR with PD153035 in Comma D cells did not prevent activation of Erk1/Erk2 by EGF (Fig. 3G). Regardless of the presence or absence of PD153035, both Erk1 and Erk2 were strongly activated within 1 min, with increasing activity up to 5 min; however, the expression of neither Erk1 nor Erk2 was affected (Fig. 3H).

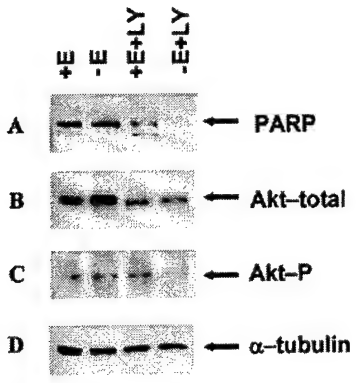

Fig. 4. Prolonged inhibition of PI3-K activity in Myc83 cells causes a decrease in Akt protein. Myc83 cells were grown in the presence or absence of EGF (+EGF, −EGF, respectively), and were treated with 50 μM LY294002 or DMSO for 48 h. (A) Apoptosis was evaluated by immunoblotting of PARP cleavage. Also, the same lysates were analyzed for the expression of Akt protein (B) and the phosphorylation status of Akt (C) by using a phospho-specific Akt (Ser473) antibody. To evaluate if the apoptotic process affects other proteins besides Akt, expression of α-tubulin (a loading control) (D) was evaluated. The results are representative of two independent assays.

Prolonged inhibition of PI3-K activity leads to apoptosis and a decrease in protein levels of both Akt and Erk1/Erk2

Recently, some evidence in the literature suggests that the Akt protein is cleaved by caspases late in the apoptotic process induced by UV irradiation, Fas ligation, and etoposide in human Jurkat cells [27]. Therefore we tested the status of the Akt protein in apoptotic Myc83 cells, following the prolonged inhibition (48 h) of PI3-K activity with 50 μM LY294002. In these experiments, we compared levels of Akt protein in LY294002-treated and untreated Myc83 cells, grown in the presence and absence of EGF. First, by evaluating cleavage of the full-length form of PARP (an early indicator of apoptosis) we found that prolonged treatment with LY294002 leads to apoptosis of Myc83 cells, regardless of the presence or absence of EGF (Fig. 4A). In the same protein lysates, apoptosis was paralleled by a prominent decrease in Akt levels (2.5-fold), independent of the presence of EGF in the growth medium (Fig. 4B). The decrease in Akt phosphorylation was detected in the sample treated with LY294002 in the absence of EGF (Fig. 4C). Furthermore, in the same samples where a decrease in the Akt protein expression and phosphorylation was detected, expression of α-tubulin protein (loading control) was unchanged (Fig. 4D), suggesting that the prolonged inhibition of the PI3-K in c-Myc-overexpressing cells specifically caused downregulation and proteolysis of the Akt protein.

To evaluate how early in the apoptotic process this downregulation of Akt was occurring, we analyzed protein lysates of Myc83 cells treated with the same concentration of LY294002 (50 μM), with and without EGF, for periods of 24 and 48 h. Again, PARP cleavage immunoblotting was used to evaluate apoptosis (Fig. 5A), and the same protein samples were further evaluated for the expression of Akt. Interestingly, as early as 24 h after treatment with LY294002, expression of Akt protein decreased (Fig. 5B), preceding the onset of apoptosis as detected by PARP cleavage (Fig. 5A). However, by 48 h, when a large percentage of the cells underwent apoptosis, as determined by PARP cleavage (Fig. 5A), the decrease in Akt protein level was more prominent (3-fold) (Fig. 5B). The mechanism responsible for decreased steady-state levels of Akt is currently under investigation.

To determine if the prolonged inhibition of PI3-K over 24 and 48 hours specifically is causing Akt downregulation, we evaluated the status of Erk1 and Erk2, Raf-1 (previously shown, together with Akt, to be cleaved late in the apoptotic process in Jurkat cells) [27], and p85 PI3-K. Similar to Akt, the expression of both Erk1 and 2 proteins decreased in the same protein lysates, in which we detected the downregulation of the Akt, following treatment with LY294002 (Fig. 5C). The decrease in Erk1 was more pronounced (2.5-fold), in comparison to Erk2 (Fig. 5C). In contrast to the reported decrease in expression of Raf-1 protein in apoptotic Jurkat cells [27], we did not detect any change in expression of Raf-1 (Fig. 5D) in apoptotic Myc83 cells. However, expression of p85 PI3-K was slightly decreased 48 h after EGF removal, and in LY294002-treated Myc83 cells, in the ab-

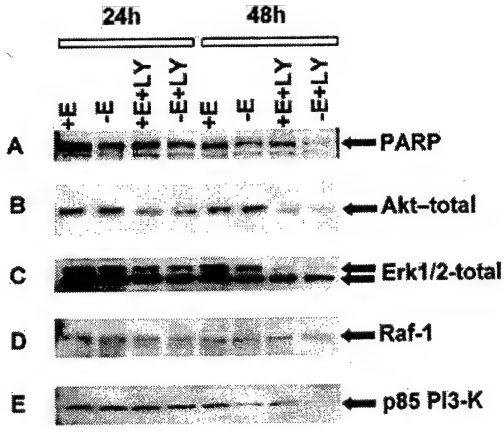

Fig. 5. Inhibition of PI3-K activity caused a decrease in expression of both Akt and Erk1/2 proteins 24 h before the onset of apoptosis. To test how early the inhibition of PI3-K activity causes the decrease in Akt expression, samples of Myc83 cells were treated with EGF (+EGF), without EGF (−EGF), and in the presence of 50 μM LY294002 (+LY) with or without EGF for 24 and 48 h. Cells were lysed and protein samples were evaluated for (A) apoptosis by immunoblotting for PARP cleavage products; (B) expression of Akt protein; (C) expression of Erk1/Erk2, (D) expression of Raf-1; (E) expression of PI3-K 85 kDa. The results are representative of two independent assays.

sence of the EGF for 48 h (Fig. 5E). The absence of Raf-1 protein downregulation further implicates the involvement of PI3-K/Akt pathway.

Downregulation of Akt protein is specific only for apoptosis induced through prolonged inhibition of PI3-K activity

To determine if the downregulation of Akt protein is simply the result of ongoing apoptosis in Myc83 cells, regardless of which of the survival pathways has been blocked, we treated Myc83 cells with 1 μM of PD153035, a specific inhibitor of the EGFR; with 10 μM of U0126 (a MEK inhibitor), and with 50 μM of LY294002 (PI3-K/Akt inhibitor) for 48 h. The samples were then evaluated for apoptosis by Hoechst staining and by PARP cleavage, as well as by immunoblotting for Akt protein expression. In the presence of EGF, only 2-3% of Myc83 cells underwent apoptosis. However, when EGF was removed, the number of apoptotic cells increased to 9% (Fig. 6A). When cells were treated with PD153035, in the presence of EGF, a total of 42% of the cells underwent apoptosis (Fig. 6A), while after removal of the EGF, 49% of the cells were apoptotic. In the presence of EGF and U0126, a total of 15% of the cells underwent apoptosis (Fig. 6A), while after removal of EGF almost 23% of the cells were apoptotic (Fig. 6A). A total of 22% of the Myc83 cells treated with EGF and LY294002 underwent apoptosis after 48 h; following EGF removal, treatment with LY294002 caused 33% of the cells to apoptose (Fig. 6A). In addition, we treated a separate set of Myc83 cells with the same inhibitors, under the same conditions for 48 h, and then lysed the cells for PARP protein analysis by immunoblotting. As expected, the results of PARP cleavage (Fig. 6B) confirmed the apoptosis results from the Hoechst staining (Fig. 6A). Interestingly, only those cells treated with an inhibitor of PI3-K (LY294002) for 48 h exhibited a significant decrease (2-fold) in the expression of the Akt (Fig. 6C). However, regardless of the apoptosis occurring in cells treated with other pharmacological inhibitors (PD153035 and U0126), the expression of Akt protein was not affected (Fig. 6C). When the Myc83 cells were treated with 60 ng/ml EGF, apoptosis induced by PD153035 treatment was inhibited up to ~70% as measured by Hoechst staining (data not shown). This shows the ability of EGF to rescue apoptosis of Myc83 cells.

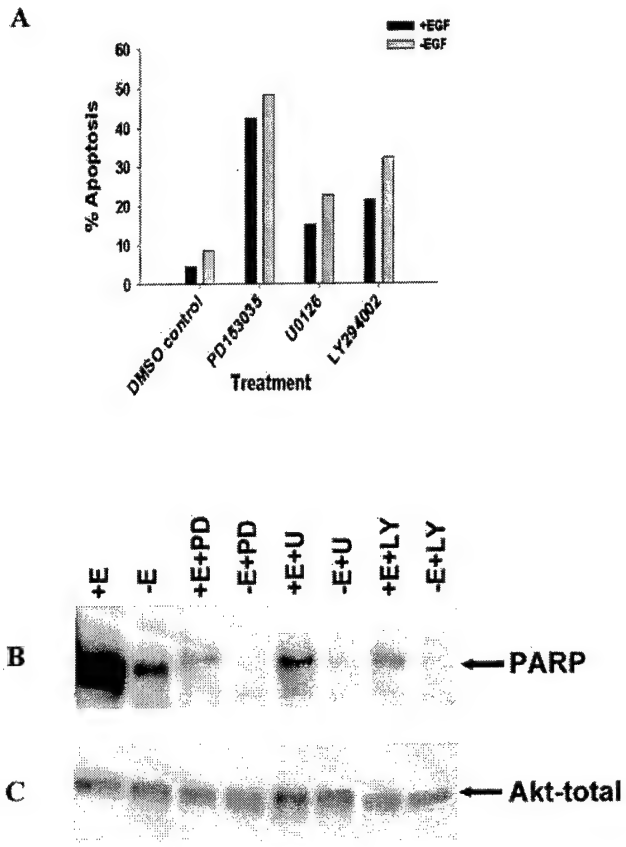

Fig. 6. Downregulation of Akt protein is specific for apoptosis induced by the inhibition of PI3-K activity. Myc83 cells were grown in IMEM medium with or without 10 ng/ml EGF, and in combination with 1 μM PD153035, 10 μM U0126, or 50 μM LY294002 for 48 h. Apoptosis was evaluated by (A) Hoechst staining (a total of 500 cells were counted) and (B) immunoblotting of PARP cleavage. (C) Akt expression was evaluated by immunoblotting using antibody-recognizing Akt independent of phosphorylation status. The results are representative of two independent assays.

Constitutively activated Akt protects MMECs overexpressing c-Myc from apoptosis

To further confirm the potential importance of Akt to deliver a survival signal downstream of EGFR, we retrovirally transduced constitutively activated Akt (Myr-Akt) into the Myc83 cells. In contrast to the parental Myc83 cells, previously shown to undergo apoptosis within 48 h of the removal of EGF and serum, Myc83-Myr-Akt cells were highly resistant to apoptosis on removal of EGF and serum from the growth medium, for up to 5 days (data not shown). The apoptotic response was evaluated by measuring PARP cleavage (Fig. 7A). PARP cleavage was detected in apoptotic pBabe-Myc83 cells treated with PD153035 for 48 h, while in Myc83-Myr-Akt cells, treated under the same conditions for 48 h, cells were protected from c-Myc-mediated apoptosis regardless of the presence of EGF or PD153035 (Fig. 7B).

Furthermore, the apoptotic profile of Myc83-Myr-Akt cells was additionally evaluated by Hoechst staining following treatment with 1 μM PD153035, 10 μM U0126, and 50 μM LY294001 for 48 h. Again, constitutively active Akt in Myc83-Myr-Akt cells almost completely protected the cells from apoptosis, resulting from the inhibition of EGFR kinase activity by PD153035 (Fig. 7C). After treatment with PD153035, in the presence of EGF, only 8% of the Myc83-Myr-Akt cells underwent apoptosis, in comparison to 42.4%

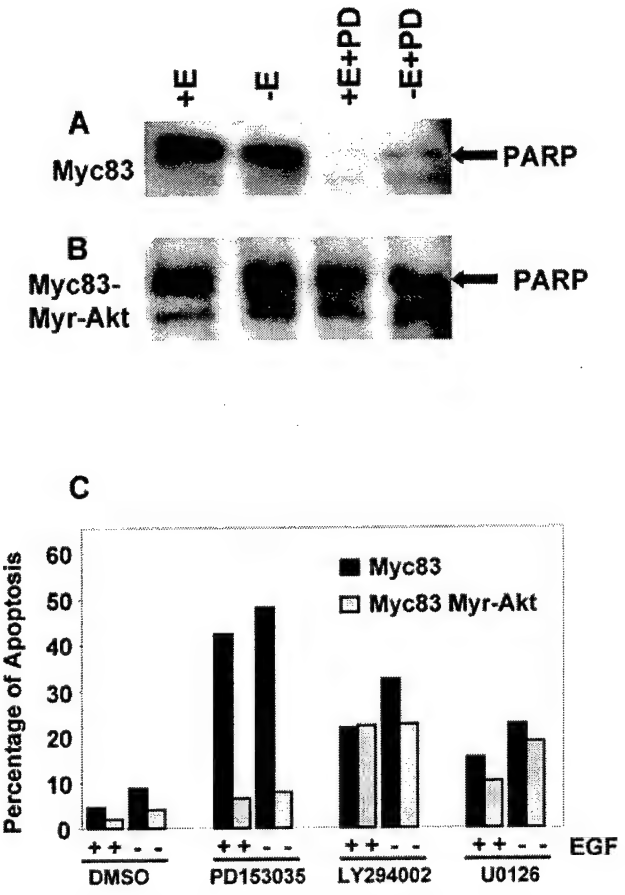

Fig. 7. Constitutively activated Akt protects MMECs overexpressing c-Myc from apoptosis. Myc83 (A) and Myc83-Myr-Akt (B) cells were grown in IMEM medium with or without EGF, and in combination with 1 μM PD153035 for 48 h. Apoptosis was analyzed by immunoblotting of PARP cleavage and Hoechst staining (C). The results are representative of two independent assays.

of Myc83 control cells (Fig. 7C). After the removal of EGF, in the presence of PD153035, a similar percentage of Myc83-Myr-Akt cells underwent apoptosis (7.4%), compared with 48.4% of control Myc83 cells in the same experiments. These data suggest that, despite the prolonged inhibition of EGFR kinase activity, the presence of constitutively activated Akt provides full protection from apoptosis in c-Myc-overexpressing MMECs.

Interestingly, constitutively activated Akt protected from apoptosis only 27% of the Myc83-Myr-Akt cells, in comparison to 22% of Myc83 in the presence of EGF plus the inhibitor of the PI3-K pathway (LY294002). Under the same conditions (in the presence of LY294002), when EGF was removed, only 29% of Myc83-Myr-Akt cells underwent apoptosis, compared with 32.5% of Myc83 cells. This result is not surprising, due to the fact that the myristolyation signal has been shown to decrease following prolonged inhibition of PI3-K activity in fibroblasts, leading to a decrease in Akt activity [24]. Also, because we detected proteolysis of endogenous Akt protein under the same conditions of prolonged inhibition of PI3-K activity for 48 h (Fig. 7C), Myr-Akt might be targeted to proteolysis as well. As expected, the constitutively activated Akt in Myc83-Myr-Akt cells did not provide significant protection from apoptosis induced by prolonged inhibition of the MEK/Erk pathway with U0126 (Fig. 7C).

Mechanism by which constitutively activated Akt inhibits apoptosis in c-Myc-overexpressing cells involves upregulation of Bcl-x_L

In fibroblasts and hematopietic cells, activated Akt has been shown to inhibit apoptosis, mostly through mechanisms involving inhibition of the pro-apoptotic Bad protein [8,12] caspase 9 [13] and inhibition of release of cytochrome c [24]. However, little information is available on the mechanism whereby Akt inhibits apoptosis in MMECs

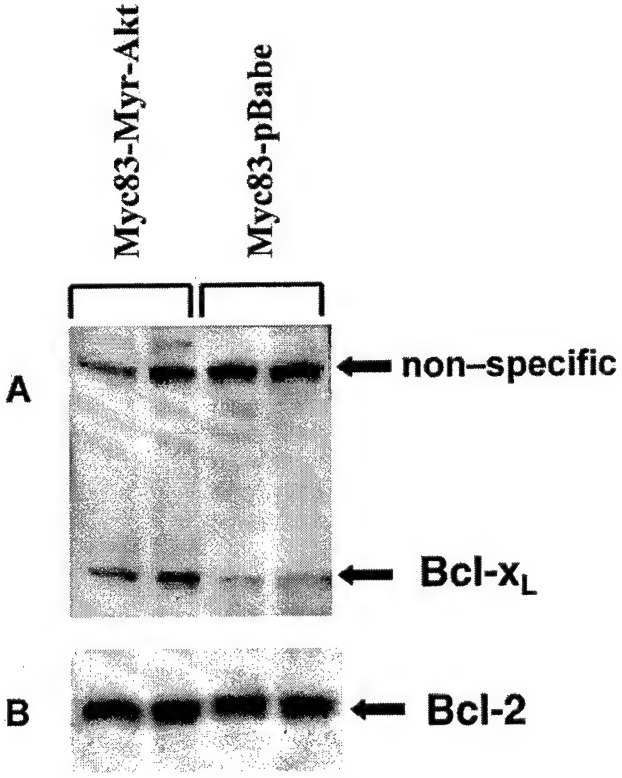

Fig. 8. Mechanism by which Akt protects MMECs from apoptosis involves the upregulation of Bcl-x_L. (A) Myc83 and Myc83-Myr-Akt were grown in IMEM medium containing 2.5% FCS, 10 ng/ml EGF (UBI), and 5 μ/ml insulin (Biofluids) Rockville, MD, USA for 48 h. Cells were lysed and protein samples analyzed by immunoblotting for the expression of Bcl-x_L protein. To increase the accuracy of the loading the same samples were loaded twice. (B) The same lysates were analyzed for Bcl-2 expression by immunoblotting. The results are representative of three independent assays.

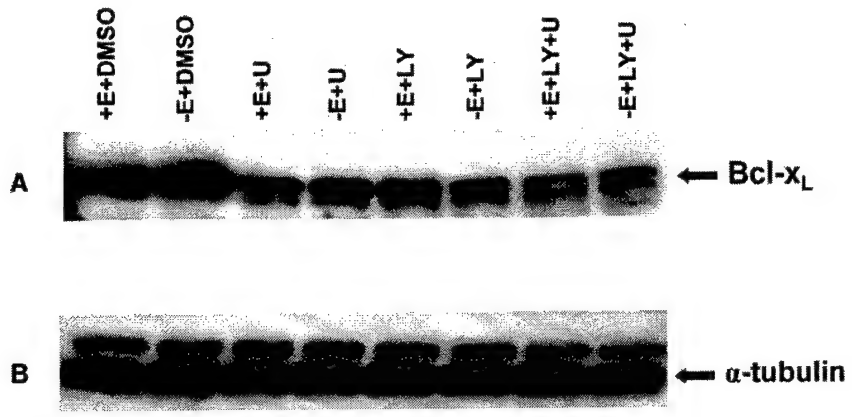

Fig. 9. Effects of pharmacological inhibitors of PI3-K/akt and MEK/Erk1/2 on Bcl-x_L protein expression in MMECs. (A) Effects of MEK inhibitor U0126 (10 μM) and PI3-K/Akt kinase inhibitor LY294002 (50 μM) on Bcl-x_L expression were measured by immunoblotting. Myc83 cells were treated with IMEM medium containing EGF and DMSO, and DMSO without EGF, and with pharmacological inhibitors with and without EGF for 48 h. Cells were lysed and protein samples were analyzed for expression of the Bcl-x_L protein. (B) The blots were reprobed with α-tubulin antibody to demonstrate equal loading. The results are representative of two independent assays.

and human breast cells and the mechanism by which Akt inhibits c-Myc-mediated apoptosis in general.

In our previous work we observed that one of the molecules upregulated by activation of EGFR in Myc83 cells is the prosurvival Bcl-x_L protein, at both the mRNA and protein levels [4]. Therefore, to explore if the mechanism whereby activated EGFR inhibits c-Myc-induced apoptosis involves Akt in our model, we analyzed the status of Bcl-x_L in parental Myc83, pBabe-Myc83, and Myc83-Myr-Akt cells grown in the presence of complete medium. Western blot analysis detected a significant, twofold increase (P = 0.005) in Bcl-x_L protein levels in Myc83-Myr-Akt cells, in comparison to Myc-83 cells transfected with vector only to pBabe-Myc83 cells (Fig. 8A).

In addition, we analyzed expression of the pro-survival Bcl-2 (Fig. 8B), 14-3-3 (data not shown), and other survival molecules, and no changes in expression were detected. Furthermore, we evaluated expression of the pro-apoptotic Bad protein, a known target of Akt. Although Bad expression levels did not differ between Myc83 and Myc-Myr-Akt cells (data not shown), it was difficult to determine the phosphorylation status of endogenous Bad in these cells by commercially available antibodies.

Effects of pharmacological inhibitors of PI3-k/Akt and Mek/Erk on Bcl-x_L protein expression

To test the role of both PI3-K/Akt and MEK/Erk1/2 pathways in the regulation of Bcl-x_L expression, we evaluated the effects of pharmacological inhibitors of these pathways on Bcl-x_L protein expression, as determined by immunoblotting. These experiments were done after 48 h of incubation with the respective compound. As shown in Fig. 9A, treatment with the MEK inhibitor U0126 in the presence (P = 0.016) and absence (P = 0.06) of EGF caused a twofold decrease in Bcl-x_L protein expression. Similarly, inhibition of the PI3-K/Akt pathway with LY294002 was associated with twofold downregulated Bcl-x_L protein expression, particularly when EGF had been removed from growth medium (P = 0.04) However, treatment with a combination of LY294002 and U0126 caused a highly significant threefold decrease in protein levels of Bcl-x_L in the presence of EGF (P = 0.003) and absence of EGF (P = 0.02). This indicates the importance of both pathways in regulation of Bcl-x_L expression. To test equal loading, blots were probed with antibody for α-tubulin (Fig. 9B).

Dominant-negative Akt and Erk1 effect the expression of Bcl-x_L

To obtain further independent evidence for the requirement of Akt and Erk signaling for expression of Bcl-x_L in Myc83, we transiently expressed DN-Akt and DN-Erk1. Similar to the PI3-K/Akt inhibitor LY294002, overexpressed DN-Akt induced Myc83 spontaneous apoptosis over a 48-h period (data not shown), and caused a significant ~40% reduction (P = 0.016) in the expression of Bcl-x_L protein (Figs. 10A and C). However, the reduction of Bcl-x_L protein expression in samples transfected with DN-Erk1 was only ~20% (P = 0.06) (Figs. 10A and C) in comparison to vector control. The effects of DN-Erk1 were less prominent in comparison to those of the specific MEK inhibitor U0123. To test for equal loading samples blots were reprobed with antibody for α-tubulin (Fig. 10B).

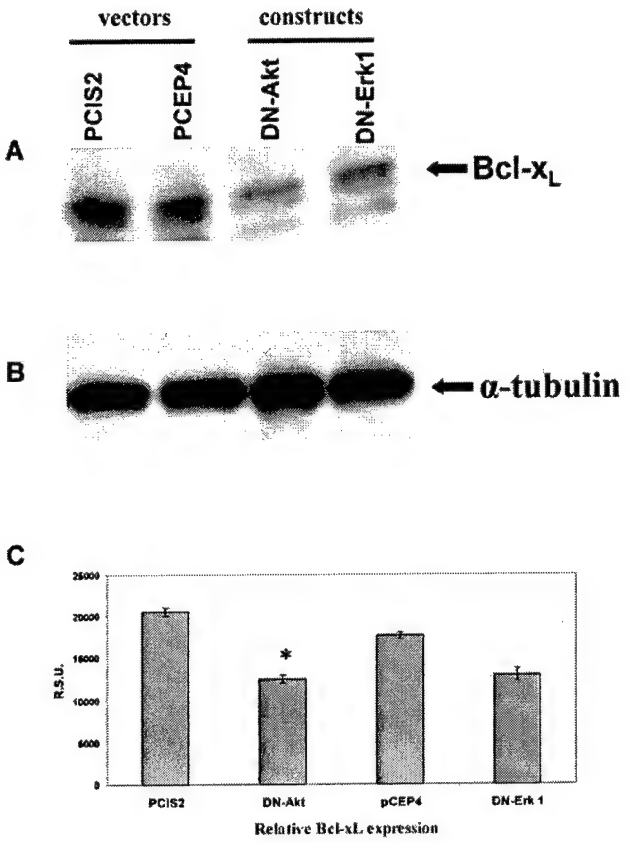

Fig. 10. Overexpression of DN-Akt and Erk1 in MMECs caused a reduction in Bcl-x_L protein. (A) Western immunoblotting analysis for expression of Bcl-x_L protein in Myc83 cells transiently transfected with vector controls (PCIS2 and pCEP4) and DN-Akt and DN-Erk1. (B) The equality of the loading was determined by reprobing blots with α-tubulin antibody. (C) R.S.U., relative scan units (with standard deviations indicated), are used to express the densitometrically measured Bcl-x_L signal to the α-tubulin in each sample. The results are representative of two independent assays. *, significantly different.

Discussion

Increased activation of EGFR [28–31] and dysregulated expression of c-Myc [1,2] are both commonly observed in human breast cancers. We previously described the dramatic interaction of these two tumor-associated aberrations in a bitransgenic model of human mammary cancer. Our studies showed that EGFR strongly suppressed c-Myc-mediated apoptosis by pro-survival signaling [3]. Signaling pathways linking EGFR to cellular survival in the context of inhibition of the pro-apoptotic state induced by c-Myc are not well defined in either mouse mammary or human breast cancer epithelial cells. We now provide evidence that EGF activates Akt/PKB and Erk1/2 in MMECs that overexpress c-Myc, and that both pathways inhibit c-Myc-mediated apoptosis, with Akt providing stronger survival signaling. These results may be specific for epithelial cells because in fibroblasts, EGF did not cause significant activation of Akt, nor was it able to protect cells from apoptosis [8].

Akt has been shown previously to inhibit apoptosis in fibroblasts, neuronal cells, hematopoietic cells, and some epithelial cells [8,9], but its role in apoptotic inhibition of mouse mammary and human breast cancer cells overexpressing c-Myc is unexplored. Recently, EGF has been reported to activate Akt in rat fetal hepatocytes [32] and in two human breast cancer cell lines, MCF-7 and T47D [33]. Our results are among the first demonstrating the importance of Akt activation in conveying survival signals downstream of EGFR in MMECs overexpressing c-Myc.

In the current study we show that constitutively activated Akt inhibits apoptosis, while upregulating protein levels of the pro-survival molecule Bcl-x_L. Furthermore, inhibition of PI3-K/Akt and MEK/Erk pathways by pharmacological inhibitors led to a significant, threefold decrease in Bcl-x_L protein expression, indicating the importance of both pathways in the regulation of Bcl-x_L protein. However, based on the results obtained with DN-Akt and DN-Erk1, it appears that Akt might have a more important role in the regulation of Bcl-x_L. This finding is consistent with our previous data, where we showed that EGF delivers survival signaling in these same c-Myc-overexpressing MMECs in association with upregulation of Bcl-x_L, at both the mRNA and protein levels [4]. Similarly, in mouse hepatocytes, EGF exerts its anti-apoptotic action partially through upregulation of Bcl-x_L [34], and EGF receptor signaling inhibits keratinocyte apoptosis through increased expression of endogenous mRNA and protein levels of Bcl-x_L [35]. However, it is not clear which signaling molecules downstream of EGFR are involved in the upregulation of Bcl-x_L. Our results here are the first indication that Bcl-x_L could be upregulated by Akt in models of human breast cancer. Currently, we are in the process of further investigating this finding in an attempt to elucidate the mechanism by which Akt upregulates Bcl-x_L.

Based on our work it seems that, contrary to what has been found in fibroblasts, Akt can regulate Bcl-x_L expression in mouse mammary epithelial cells. Similarly, it has been reported that Akt promotes T-lymphocyte survival through enhanced expression of Bcl-x_L protein in vivo, without affecting its mRNA level [15]. In rat pheochromocytoma (PC12) cells, Akt upregulates Bcl-2 expression through the c-AMP response element-binding protein [16], but any effect on Bcl-x_L has not been addressed. Finally, recent reports indicate that PI3-K activity can induce Bcl-x_L expression, at both the mRNA and protein levels in Baf-3 cells, but this study does not implicate Akt directly [36].

Bcl-x_L has been shown to insert into the mitochondrial membrane and form ion channels [37], directly controlling

mitochondrial cytochrome c release [38]. In both prior studies, the mitochondrial membrane potential was affected, and apoptosis was inhibited [37,38]. In some studies, Bcl-x_L has been shown to interact with caspase 9 and with apoptotic protease-activating factor (APAF-1), resulting in apoptotic inhibition [39]. It has been shown in hematopoetic cells that Akt and Bcl-x_L promote interleukin-3-independent survival through distinct effects on mitochondrial physiology [40]; however, it is not clear if this is the case in epithelial cells, including mouse mammary and breast cancer cells. The mechanism by which Akt, Erk, and upregulated Bcl-x_L contribute to apoptosis inhibition in our model is currently under investigation. There is a possibility, currently under exploration, that a twofold upregulation of Bcl-x_L protein could play a role in inhibition of cytochrome c release in c-Myc-overexpressing MMECs. In fibroblasts, the release of cytochrome c was reported to be involved in c-Myc-induced apoptosis [41].

Although the role of Akt in human breast cancer and MMECs is not completely understood, the role of Erk/MAPK in transformation of mouse mammary cells and in cancerous human breast tissue is firmly established in the literature [42]. Here we found that EGF activates Erk and Akt within a similar time frame. In addition, we have found that, although potentially less potent than PI3-K/Akt, the MAPK/Erk pathway also delivers a survival signal in Myc83 cells. Supporting this finding, Erk has been reported to deliver a survival signal in human chondrocytes [43] and in human neutrophils [44], and it was shown to cooperate with Akt in delivering survival signal to MCDK cells [26]. Recently, it has been also shown that one of the ways in which the MEK/Erk pathways induce survival in keratinocytes involves expression of Bcl-x_L [28]. Based on our results here, we predict the interaction of Akt and Erk in delivering survival through common targets in MMECs. One such target for both pathways could be Bcl-x_L.

In our study we have also noted a PI3-K-dependent downregulation of both Akt and Erk protein levels, resulting from the prolonged (24–48 h) inhibition of PI3-K activity. Although it was previously reported that Akt is targeted by caspases during Fas-induced apoptosis of human Jurkat cells and UV-irradiated U937 cells [27], it seems that caspases are not targeting Akt in Myc83 cells. Presently, it is not clear what are the reasons for these differences, but it could be potentially explained by the species differences. It seems that in Myc83 cells, the mechanism responsible for cleavage of both Akt and Erk, may not involve caspases. When we blocked the activity of EGFR and Erk/MAPK kinase, we could not detect Akt or Erk proteolysis (Fig. 6C), despite the induction of apoptosis (Figs. 6A and B). Thus, the proteolysis of Akt and Erk may be dependent on selective inhibition of PI3-K. In addition, it appears that PI3-K regulates the expression levels of Erk1 as well. The mechanism(s) of these PI3-K effects is not known at present. Our findings on Erk downregulation are consistent with a recent report, which showed that the inhibitors of PI3-K activity could block the Erk/MAPK kinase signaling pathway [45]. Also, PI3-K has been shown to control the activity of Erk/MAPK through Raf, a molecule regulating Erk function [46].

For the first time, we have demonstrated that activated Akt and Erk could be responsible for the EGFR-regulated overexpression of Bcl-x_L in models of human breast cancer. These findings may be of importance considering the unexplored role of both Akt and the Bcl-x_L in mouse mammary tumors and human breast cancer. Recently, Akt and Bcl-x_L have both been viewed as potential therapeutic targets in several human cancers and our findings could be a significant contribution. Furthermore, overexpressed Bcl-x_L protein is being considered as a prognostic marker in a few studies of breast carcinomas where its overexpression has been associated with advanced-stage disease.

Acknowledgments

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References

[1] S.J. Nass, R.B. Dickson, Defining a role for c-Myc in breast tumorigenesis, Breast Cancer Res. Treat. 44 (1997) 1–22.
[2] D.J. Liao, R.B. Dickson, c-Myc in breast cancer, Endocr. Relat. Cancer 7 (2000) 143–164.
[3] L.T. Amundadottir, S.J. Nass, G.J. Berchem, M.D. Johnson, R.B. Dickson, Cooperation of TGF alpha and c-Myc in mouse mammary tumorigenesis: coordinated stimulation of growth and suppression of apoptosis, Oncogene 13 (1996) 757–765.
[4] S.J. Nass, M. Li, L.T. Amundadottir, P.A. Furth, R.B. Dickson, Role for Bcl-x_L in the regulation of apoptosis by EGF and TGFβ in c-myc overexpressing mammary epithelial cells, Biochem. Biophys. Res. Commun. 227 (1996) 248–256.
[5] R.A. Roberts, N.H. James, S.C. Cosulich, The role of protein kinase B and mitogen-activated protein kinase in epidermal growth factor and tumor necrosis factor alpha-mediated rat hepatocyte survival and apoptosis, Hepatology 31 (2000) 420–427.
[6] M. Sibilia, A. Fleischmann, A. Behrens, L. Stingl, J. Carroll, F.M. Watt, J. Schlessinger, E.F. The EGF receptor provides an essential

survival signal for SOS-dependent skin tumor development Wagner, Cell 102 (2000) 211–220.
[7] J. Downward, Mechanisms and consequences of activation of protein kinase B/Akt, Curr. Opin. Cell Biol. 10 (1998) 262–267.
[8] E.S. Kandel, N. Hay, The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB, Exp. Cell Res. 253 (1999) 210–229.
[9] S.R. Datta, A. Brunet, M.E. Greenberg, Cellular survival: a play in three Akts, Genes Dev. 13 (1999) 2905–2927.
[10] P.J. Coffer, J. Jin, J.R. Woodgett, Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation, Biochem. J. 335 (1998) 1–13.
[11] J. Okano, I. Gaslightwala, M.J. Birnbaum, A.K. Rustgi, H. Nakagawa, Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation, J. Biol. Chem. 275 (2000) 30934–3094.
[12] S.R. Datta, H. Dudek, X. Tao, S. Masters, H. Fu, Y. Gotoh, M.E. Greenberg, Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery, Cell 91 (1997) 231–241.
[13] M.H. Cardone, N. Roy, H.R. Stennicke, G.S. Salvesen, T.F. Franke, E. Stanbridge, S. Frisch, J.C. Reed, Regulation of cell death protease caspase-9 by phosphorylation, Science 282 (1998) 1318–1321.
[14] A. Brunet, A. Bonni, M.J. Zigmond, M.Z. Lin, P. Juo, L.S. Hu, M.J. Anderson, K.C. Arden, J. Blenis, M.E. Greenberg, Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor, Cell 96 (1999) 857–868.
[15] R.G. Jones, M. Parsons, M. Bonnard, V.S. Chan, W.C. Yeh, J.R. Woodgett, P.S. Ohashi, Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels *in vivo*, J. Exp. Med. 191 (2000) 1721–1734.
[16] S. Pugazhenthi, A. Nesterova, C. Sable, K.A. Heidenreich, L.M. Boxer, L.E. Heasley, J.E. Reusch, Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein, J. Biol. Chem. 275 (2000) 10761–10766.
[17] M.L. Kuo, S.E. Chuang, M.T. Lin, S.Y. Yang, The involvement of PI 3-K/Akt-dependent up-regulation of Mcl-1 in the prevention of apoptosis of Hep3B cells by interleukin-6, Oncogene 20 (2001) 677–685.
[18] J.A. Romashkova, S.S. Makarov, NF-kappaB is a target of AKT in anti- apoptotic PDGF signaling, Nature 401 (1999) 86–90.
[19] S. Pianetti, M. Arsura, R. Romieu-Mourez, R.J. Coffey, G.E. Sonenshein, Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN, Oncogene 20 (2001) 1287–1299.
[20] L. Moro, M. Venturino, C. Bozzo, L. Silengo, F. Altruda, L. Beguinot, G. Tarone, P. Defilippi, Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival, EMBO J. 17 (1998) 6622–6632.
[21] F. Walker, A. Kato, L.J. Gonez, M.L. Hibbs, N. Pouliot, A. Levitzki, A.W. Burgess, Activation of the Ras/mitogen-activated protein kinase pathway by kinase-defective epidermal growth factor receptors results in cell survival but not proliferation, Mol. Cell Biol. 18 (1998) 7192–7204.
[22] J. Hayakawa, M. Ohmichi, H. Kurachi, Y. Kanda, K. Hisamoto, Y. Nishio, K. Adachi, K. Tasaka, T. Kanzaki, Y. Murata, Inhibition of BAD phosphorylation either at serine 112 via extracellular signal-regulated protein kinase cascade or at serine 136 via Akt cascade sensitizes human ovarian cancer cells to cisplatin, Cancer Res. 60 (2000) 5988–5994.
[23] S.M. Campbell, M.M. Taha, D. Medina, J.M. Rosen, A clonal derivative of mammary epithelial cell line COMMA-D retains stem cell characteristics of unique morphological and functional heterogeneity, Exp. Cell Res. 177 (1988) 109–121.
[24] S.G. Kennedy, E.S. Kandel, T.K. Cross, N. Hay, Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria, Mol. Cell Biol. 19 (1999) 5800–5810.
[25] D.A. Cross, D.R. Alessi, P. Cohen, M. Andjelkovich, B.A. Hemmings, Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B, Nature 378 (1995) 785–789.
[26] A. Danilkovitch, S. Donley, A. Skeel, E.J. Leonard, Two independent signaling pathways mediate the antiapoptotic action of macrophage-stimulating protein on epithelial cells, Mol. Cell Biol. 20 (2000) 2218–2227.
[27] C. Widmann, S. Gibson, G.L. Johnson, Caspase-dependent cleavage of signaling proteins during apoptosis: a turn-off mechanism for anti-apoptotic signals, J. Biol. Chem. 273 (1998) 7141–7147.
[28] M. Jost, T.M. Huggett, C. Kari, L.H. Boise, U. Rodeck, Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-x_L expression through a MEK-dependent pathway, J. Biol. Chem. 276 (2001) 6320–6326.
[29] J. Mendelsohn, J. Baselga, The EGF receptor family as targets for cancer therapy, Oncogene 19 (2000) 6550–6565.
[30] R. Soares, M.B. Pereira, C. Silva, I. Amendoeira, R. Wagner, J. Ferro, F.C. Schmitt, Expression of TGF-alpha and EGFR in breast cancer and its relation to angiogenesis, Breast J. 6 (2000) 171–177.
[31] N. Prenzel, E. Zwick, M. Leserer, A. Ullrich, Tyrosine kinase signaling in breast cancer: epidermal growth factor receptor: convergence point for signal integration and diversification, Breast Cancer Res. 2 (2000) 184–190.
[32] I. Fabregat, B. Herrera, M. Fernandez, A.M. Alvarez, A. Sanchez, C. Roncero, J.J. Ventura, A.M. Valverde, M. Benito, Epidermal growth factor impairs the cytochrome C/caspase-3 apoptotic pathway induced by transforming growth factor beta in rat fetal hepatocytes via a phosphoinositide 3-kinase-dependent pathway, Hepatology 32 (2000) 528–535.
[33] S. Gibson, S. Tu, R. Oyer, S.M. Anderson, G.L. Johnson, Epidermal growth factor protects epithelial cells against Fas-induced apoptosis: requirement for Akt activation, J. Biol. Chem. 274 (1999) 17612–17618.
[34] L. Musallam, C. Ethier, P.S. Haddad, M. Bilodeau, Role of EGF receptor tyrosine kinase activity in antiapoptotic effect of EGF on mouse hepatocytes, Am. J. Physiol. Gastrointest. Liver Physiol. 280 (2001) 1360–1369.
[35] S.W. Stoll, M. Benedict, R. Mitra, A. Hiniker, J.T. Elder, G. Nunez, EGF receptor signaling inhibits keratinocyte apoptosis: evidence for mediation by Bcl-x_L, Oncogene 16 (1998) 1493–1499.
[36] Y. Leverrier, J. Thomas, A.L. Mathieu, W. Low, B. Blanquier, J. Marvel, Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells, Cell Death Differ. 6 (1999) 290–296.
[37] M.G. Vander Heiden, X.X. Li, E. Gottleib, R.B. Hill, C.B. Thompson, M. Colombini, Bcl-x_L promotes the open configuration of the voltage-dependent anion channel and metabolite passage through the outer mitochondrial membrane, J. Biol. Chem. 276 (2001) 19414–19419.
[38] M.G. Vander Heiden, N.S. Chandel, E.K. Williamson, P.T. Schumacker, C.B. Thompson, Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria, Cell 91 (1997) 627–637.
[39] G. Pan, K. O'Rourke, V.M. Dixit, Caspase-9, Bcl-x_L, and Apaf-1 form a ternary complex, J. Biol. Chem. 273 (1998) 5841–5845.
[40] D.R. Plas, S. Talapatra, A.L. Edinger, J.C. Rathmell, C.B. Thompson, Akt and Bcl-xL promote growth factor-independent survival through distinct effects on mitochondrial physiology, J. Biol. Chem. 276 (2001) 12041–12048.
[41] P. Juin, A.O. Hueber, T. Littlewood, G. Evan, c-Myc-induced sensitization to apoptosis is mediated through cytochrome c release, Genes Dev. 13 (1999) 1367–1381.

[42] C. Xing, W. Imagawa, Altered MAP kinase (ERK1, 2) regulation in primary cultures of mammary tumor cells: elevated basal activity and sustained response to EGF, Carcinogenesis 20 (1999) 1201–1208.

[43] M. Shakibaei, G. Schulze-Tanzil, P. de Souza, T. John, M. Rahmanzadeh, R. Rahmanzadeh, H.J. Merker, Inhibition of mitogen-activated protein kinase kinase induces apoptosis of human chondrocytes, J. Biol. Chem. 276 (2001) 13289–13294.

[44] K. Suzuki, T. Hasegawa, C. Sakamoto, Y.M. Zhou, F. Hato, M. Hino, N. Tatsumi, S. Kitagawa, Cleavage of mitogen-activated protein kinases in human neutrophils undergoing apoptosis: role in decreased responsiveness to inflammatory cytokines, J. Immunol. 166 (2001) 1185–1192.

[45] K. Suzuki, K. Takahashi, Anchorage-independent activation of mitogen-activated protein kinase through phosphatidylinositol-3 kinase by insulin-like growth factor I, Biochem. Biophys. Res. Commun. 272 (2000) 111–115.

[46] S.L. Sutor, B.T. Vroman, E.A. Armstrong, R.T. Abraham, L.M. Karnitz, A phosphatidylinositol 3-kinase-dependent pathway that differentially regulates c-Raf and A-Raf, J. Biol. Chem. 274 (1999) 7002–7010.

SIGNALING TO THE BREAST

Danica Ramljak[1] and Robert B. Dickson[1]

[1] *Department of Oncology, Lombardi Cancer Center, Georgetown University, Washington D.C. 20007*

The address: The Research Building, Rm. 417, Georgetown University, 3970 Reservoir Rd., NW, Washington D.C. 20007

Danica Ramljak
Tel.: 202-687-2277
Fax: 202-687-7505
ramljakd@georgetown.edu

Robert B. Dickson
Tel.: 202-687-3770
Fax: 202-687-7505
dicksonr@georgetown.edu

Deregulated cell and tissue growth is a defining feature of all neoplasms, both benign and malignant. Malignant neoplasms have the capacity to invade normal tissues, to induce the development of a local vasculature, to metastasize and to grow at distant body sites. Deregulated growth occurs as a result of perturbed signal transduction. Signal transduction pathways include in their broadest sense, all biochemical cellular pathways that modulate or alter cellular behavior or function. Consequently, cancers do not necessarily arise as a result of an increased rate of cellular proliferation. Rather, carcinogenesis is a combination of defects in cell-cycle progression (cellular division), immortalization, genomic instability, programmed cell death (apoptosis) cell-cell and cell-substrate adhesion and angiogenesis. During normal embryonic development and in adult life, signaling needs to be precisely coordinated and integrated at all times, because properly regulated differentiation signals are critical for preventing oncogenesis.

Studies of the normal and neoplastic breast, during last several decades of the twentieth century, focused on identification of the mechanisms of action of estrogen and progesterone at the local tissue level, in the normal breast, and early promotion and later progression to malignancy. Breast tissue regulation by these hormones is modulated, in rather complex fashion, by autocrine and paracrine growth factors and by a variety of transcription factors (such as coactivators and corepressors), controlling epithelial cellular differentiation, epithelial cell-cell and cell-stromal adhesion. A large body of breast cancer research has been focused on understanding the complex interactions among growth factors, deregulated growth-regulatory genes (oncogenes or protooncogenes) in mediating or modulating endocrine steroid action in breast cancer.

A second important topic has been the involvement of growth factors in facilitating malignant progression of the disease. One area of research has examined defective tumor host

interactions, resulting in aberrant stromal collagen synthesis (desmoplasia), epithelial cell invasion and vascular infiltration (angiogenesis) to promote distant metastasis. Also, studies have found that certain growth factors may suppress the host immune response to the tumor and may influence a tumor's response to therapy.

In the past few years, significant progress has been made in understanding the roles of different signaling molecules in normal and malignant breast. However, for the purpose of this chapter we will focus only on several major molecules and pathways, such as the epidermal growth factor family (EGF), TGFβ family/Smads, and some other families of growth factors that have been shown to be important in breast signaling. In addition, three major proliferation/survival molecules/pathways Mek/Erk, PI3-K/Akt and Stats will be discussed. We will not address the roles of cell adhesion molecules, nor steroid hormones in signaling; other reviews covering these topics are available [1-3].

I. SIGNALING MOLECULES: A CLASS OF GROWTH FACTORS

A. Epidermal Growth Factor Family

Two major classes of structurally and functionally distinct transforming growth factor families were initially characterized by the prototypic transforming growth factor α (TGFα) and transforming growth factor β (TGFβ). TGFα is closely homologous to epidermal growth factor (EGF), and both bind to the common EGF receptor (EGFR) [4]. EGFR is tyrosine–kinase that upon the activation stimulates several cellular responses including survival, proliferation, motility and differentiation [4]. The EGF family members that bind to EGFR are: EGF, TGFα, amphiregulin (AR, a heparin-binding factor), heparing-binding EGF (HbEGF), epiregulin and β-cellulin [5]. Cripto (CR-1) is another EGF family member with a demonstrated role in

embryogenesis, mammary gland development and it has been overexpressed in several human tumors [6-7]. Recently, the nature of its receptor has been identified as an ALK4, a type I serine/threonine kinase receptor for activin. CR-1 binds to the cell surface ALK4 that is expressed on mammalian epithelial cells [8]. More recently, molecules known as *heregulins* (human) and NDFs (*neu* differentiation factor, from rat) were cloned and identified [9-10]. Neuregulins is a widely used common term for both NDFs and *heregulins*. Unlike most of the other growth factors, EGF family members that bind directly to EGFR, neuregulins bind to two other EGF family members termed c-Erb-B3 and c-Erb-B4 which are structurally related [11]. While c-Erb-B4 is a tyrosine kinase, c-Erb-B3 is kinase defective. A fourth member c-Erb-B2 (HER2/neu) binds no ligand and is activated only through heterodimeric interaction with other family members. c-Erb-B2 is particularly important as an oncogene in breast cancer [12]. All four members of the EGFR family (Table 1) and most of the EGFR-related growth factors have been shown to play a role in the tumor growth, development and progression of human breast cancer.

Recently, mouse models have provided important corroboration and new insights, regarding the role of EGF family members in mammary gland development. The view emerging from recent work is that signaling by EGFR-and possibly c-Erb-B2 is critical for ductal outgrowth in pubescent gland, while signaling by c-Erb-B2, c-Erb-B3 and c-Erb-B4 is important for alveolar morphogenesis and lactation. Presently, it is unclear whether EGFR has a significant role in the latter processes. In a recent study only EGFR and c-Erb-B2 proteins appeared to play a role in the developing virgin gland [11], while all four members of the receptor family were expressed and maximally phosphorylated during pregnancy and lactation. Results of several studies indicate that members of EGF family could act local mediators of ductal morphogenesis.

Analysis of triple null mice lacking EGF, TGFα, and AR, alone or in various combinations, suggested a physiological role for AR ligand, a very surprising result in light of this ligand's reduced affinity for EGFR [13]. These results from animal models demonstrate a requirement for activation of EGFR by AR in ductal development in the adolescent mammary gland. Elongating ducts of adolescent mice contained high levels of the AR transcript, whereas expression was not detected in surrounding fibroblasts or fat cells, indicating that the epithelium is the principal source of AR in the developing mammary gland [13]. In contrast, EGFR protein and mRNA were detected in all cellular compartments, including epithelial cells, fibroblasts and adipocytes. Thus, ductal outgrowth could be stimulated by autocrine and/or paracrine activation of EGFR. However, available evidence supports a paracrine model. Furthermore, while a role of EGFR in mammary gland development is obvious, until recently it was less clear whether this receptor also functions in alveolar morphogenesis and lactation. Currently, there is evidence supporting such a role. Overexpression of TGFα from WAP-driven transgene induced precocious alveolar development and delayed involution in transgenic mice [14], suggesting that these processes might be normally regulated by EGFR. EGFR levels, and its phosphorylation, were also shown to coordinately peak in late pregnancy and lactation [15]. In addition, aberrant epithelial differentiation and impaired lobular expansion were observed in the mammary gland of the AR knockout mice, a phenotype that was aggravated by additional loss of EGF and TGFα [13].

Steroid-growth factor interactions have been studied in human mammary tissue only in the contex of malignant epithelium, although they are almost certainly crucial as well in the regulation of the normal gland and in the development of cancer. In hormone responsive human breast cancer cells, estrogen-induced proliferation is accompanied by an increase in growth-

stimulatory TGFα, AR and IGF-II, modulation of IGF-binding proteins, induction of EGF and IGF-I receptors, inhibition of IGF-II and c-Erb-B2 receptors, and inhibition of TGFβ [16].

As mentioned above, the role of EGF family members in tumor onset and progression is well-established [5]. TGFα has been shown, both in cell lines *in vitro* and in experimental animal models *in vivo,* as a positive modulator of cellular transformation. These effects of TGFα are due to its effects on proliferation, survival and motility, as well as modulation of differentiation [17]. Several laboratories, including our own, previously developed and/or utilized mouse models in which the EGFR ligand, TGFα, was overexpressed in the mammary gland under control of MMTV LTR or WAP promoter [18-20]. The presence of the transgene in the mammary gland caused anomalous development: alveoli appeared precociously, and post-lactational involution was impaired, resulting in persistent epithelial structures, termed hyperplastic aveolar nodules. The mice also developed focal mammary tumors with high efficiency and short latency [18], indicating that ErbB signaling leads to neoplastic progression in this tissue. Recently, Humphreys and Hennighausen [19- 21], compared TGFα-induced mammary tumorigenesis in wild type mice versus those lacking a functional Stat5a gene. They found that the absence of Stat5a delayed hyperplasia and tumor development, and coincidentally, promoted more epithelial regression. These effects were not observed in TGFα transgenic mice containing Stat5a.

Our own studies and research of a few other laboratories have been focused on understanding of cooperation and synergy between TGFα and c-Myc that is frequently amplified and overexpressed in human breast cancer and it is a downstream effector of the c-Erb-B2 oncogene. Bitransgenic c-Myc/TGFα mice developed multiple aggressive mammary tumors with dramatically shorter latency compared to either single transgenic lineage. However, tumors were also frequently observed in bitransgenic mice [18]. These results indicate a strong synergy

between TGFα and c-Myc that could reflect the latter's ability to amplify autocrine growth circuits, including those involving EGFR, in cultured cells. In addition, our recent data indicate that there is another possibility involving cooperation between cyclin D1 and TGFα [22]. It appears that the early upregulation of cyclin D1 by TGFα might circumvent the normal ability of c-Myc to repress this cell cycle regulator, perhaps obviating the need for genetic alterations that would otherwise uncouple c-Myc and cyclin D1 during neoplastic progression. Work by Shroeder *et al.*, [20], focused on identifying genes that cooperate with TGFα in mammary tumorigenesis. Based on their results it appears that TGFα caused upregulation of the Wnt 3 gene, suggesting that the synergy between EGFR and Frizzled signaling pathways might contribute to neoplastic progression. Both Wnt and ErbB receptors can regulate β-catenin activity leading to cellular disaggregation. Therefore, it is possible to speculate that the observed synergy involves this pathway.

Besides the role of TGFα in mammary tumorigenesis, other EGFR ligands might have a role in the proliferation process. The closely related factor, EGF, has been shown to act as an oncogene–like molecule, when transfected and overexpressed in immortalized rodent fibroblasts [11]. Furthermore, it has been shown that besides TGFα, both AR and cripto-1 may be important in early stages of onset of mammary cancer [15, 23-27]. Recently, a new transgenic model of EGFR overexpression in mammary gland has been described [28]. Mammary glands of virgin mice harboring an EGFR transgene, under control of the MMTV or β-lactoglobulin (BLG) promoters, developed abnormally and displayed epithelial hyperplasias. With multiple pregnancies, dysplasias and tubular adenocarcinomas were also observed. Differentiation of mammary epithelium was perturbed in response to deregulated EGFR, as reflected by fewer alveoli developing in whole mount organ cultures.

Similarly to EGFR, overexpression of c-Erb-B2 in the transgenic mouse, or in the transgenic mammary gland after retroviral transfer, also leads to pregnancy-induced mammary tumors [11]. Some limited studies have also addressed the function of the c-Erb-B/c-Erb-B4 ligand family and neuregulins in breast cancer [11, 29].

Clearly, signaling by the ErbB network is important to the complex overall regulation of the mammary gland. It appears that all the models described above have firmly established that altered regulation or signaling of ErbB receptors and ligands can facilitate tumor promotion and/or neoplastic progression in the mammary gland. Future issues to be solved will include determination of definite roles of c-Erb-B3 and c-Erb-B4 in mammary tumorigenesis, exploration of cooperative roles and interaction of EGFR and c-Erb-B2 in carcinogenesis, and the interactions of the c-ErbB family with other oncogenes and hormones in human breast cancers. In addition, it will be of interest to establish the identity of critical intracellular signaling pathways, downstream of these receptors in human breast cancer at various steps in malignancy. Our own preliminary work and work of others indicate that both PI3/K and MEK/Erk pathway are two key survival and proliferation signaling pathways, conveying signals downstream of the EGFR and possibly other members of the family. These two pathways will be discussed later in this chapter.

B. Transforming Growth factor β family

The TGFβ family consists of several related gene products, each forming 25 kd homodimeric or heterodimeric species, found in both normal mammary epithelium and in breast cancer. Three membrane-binding proteins interact with this family of growth factors. These were initially termed receptors (type I, II and III) but type III receptors seem to be nonsignaling proteins, while the other two types (type I and II) are serine-threonine kinases, and have been shown to deliver

intracellular signals [30-34]. Four different type II receptors have been cloned, and they each may associate with one of several type II receptors. The function of type II receptors is determined on the basis of which type I receptor is recruited into dimer formation. TGFβ ligands only directly bind to type II receptors.

The epithelial-inhibitory, TGFβ family is found, in normal and malignant mammary epithelium and human milk [35]. TGFβ clearly has a negative effect on ductal epithelial proliferation and lactation in mouse mammary glands *in vivo* [36]. It is important to note that the production of TGFβ increases as breast cancer progresses; its accumulation appears to be important in the characteristic fibrous desmoplastic stroma of the disease [37], in immune suppression, and in tumor angiogenesis. It seems that, although TGFβ clearly serves as a growth-inhibitory role in the normal gland, in which it may be tumor suppressive, overproduction of TGFβ may contribute to aberrant tumor-host interactions in the later progression of breast cancer [35].

In the recent review by Wakefield and Roberts [38] on TGFβ signaling in mammary gland development and tumorigenesis, several important issues have been summarized. First, ligands of the TGFβ superfamily are unique, in that they signal through transmembrane receptor serine-threonine kinases, rather than the tyrosine kinases of many other well-known growth factors in the breast. The TGFβ receptor complex couples to a signal transduction pathway involving a novel family of proteins, the Smads. On phosphorylation, Smads translocate to the nucleus where they modulate transcriptional responses. However, TGFβ can also activate the mitogen-activated protein kinase (MAPK) 4 pathway, and the different biological responses to TGF-β depend to varying degrees on activation of either or both of these two pathways. The Smad pathway is a nexus for cross-talk with other signal transduction pathways, and for

modulation by many different interacting proteins. Despite compelling evidence that TGFβ has tumor suppressor activity in the mammary gland, neither TGFβ receptors nor Smads are genetically inactivated in human breast cancer, although receptor expression is reduced. Recent work by Xie *et al.* [39] provides new information on the role of Smads in human breast cancer. It shows that alterations of Smad signaling in human breast carcinoma are associated with poor outcome. Among 456 cases of human breast carcinoma assembled in tissue microarrays, the majority (92%) expressed Smad2, Smad2P, as well as Smad4, indicating their ability to proliferate within a microenvironment that contains bioactive TGFβ.

Although some of the roles of TGFβ in the mammary gland signaling are obvious, some of the mechanisms in which TGFβ might play a role in development of human breast cancer are poorly understood. This needs to be addressed in future research initiatives.

C. Other families of growth factors

In addition to EGFR and TGFβ growth factor families, there are at least five other growth factor stimulatory molecules found to play a role in breast cancer. These are summarized in Table 2: insulinlike growth factors (Type I and II) [40], members of the Wnt (wingless) growth factor-receptor family (Wnt-2, Wnt-3, Wnt-4, Wnt5a and Wnt-7b) [41], platelet-derived growth factors A and B [42] and the fibroblast growth factor (FGF) family [43]. Each of these growth factor classes binds to one or more specific tyrosine kinase-encoding receptors. Vascular endothelial growth factor (VEGF, a member of a different family of tyrosine kinase receptor-binding factors) [44], pleiotrophin (a developmental, neurotropic factor) [45] *HGF*, (*hepatocyte growth factor* or scatter factor) and its tyrosine kinase encoding receptor, c-Met [46], are all

produced by breast cancer. In addition, breast cancer cells, also produce the hormone prolactin [47], and mammary-derived growth factor 1 (MDGF-1) [48].

Although the roles of some of the molecules mentioned above are well established in breast signaling, much more research is needed to fully understand their role in both mammary gland development and in human breast cancer.

II. PI3-K/AKT, MEK/ERK and STATs: MAJOR PROLIFERATION/SURVIVAL MOLECULES DOWNSTREAM OF GROWTH FACTOR RECEPTORS IN BREAST

There is limited information on the signaling pathways linking EGFR and other growth factor receptors to the regulation of cellular survival in mouse and human mammary epithelial and carcinoma cells. However, studies in some non-mammary epithelial cells (hepatic carcinoma cells and keratinocytes) have identified two survival pathways downstream of EGFR: phosphatidylinositol 3-kinase (PI3-K)/Akt and extracellular signal regulated kinase (Erk1/2). In most cases, the PI3-K/Akt pathway delivers the most potent survival signals downstream of EGFR [49-50].

Akt is a serine-threonine kinase, downstream of PI3-K, which delivers strong survival signals in many cell types [51-53]. Both growth factors and integrins activate Akt through activation of PDK1 and a putative PDK2 kinase, that subsequently phosphorylate Akt at Thr308 and Ser473, respectively [54]. There are several isoforms of Akt (Akt1, Akt2 and Akt3); each has been shown to be expressed at different levels in various tissues [55]. The targets of Akt in epithelial cells, including mouse and human breast cells, include proteins involved in cell growth, metabolism and apoptosis. The Akt targets involved in apoptosis include Bad, a pro-apoptotic member of the Bcl-2 family of proteins [56], caspase 9 [57], and the forkhead

transcription factor [58]. Recently, Akt also has been reported to upregulate the expression of anti-apoptotic proteins in lymphoid cells such as Bcl-x_L [59], Bcl-2 [60] and Mcl-1 [61]. Akt activates NF-κB in both fibroblasts and epithelial cells [62-63].

Similarly to Akt, in most cell types, both growth factors and integrins are capable of activating the MAPK/Erk pathway. Of particular relevance to our studies, involving models of human breast cancer, is the fact that the MAPK/Erk pathway was previously shown to convey survival signals in response to EGF [64-65].

Recently, it has been shown that the PI3-K/Akt and the MAPK/Erk pathways can cooperate in the inhibition of Bad in some cell types [66]. However, the pro-survival targets and the interactions of EGFR-activated PI3K/Akt and MAPK/Erk pathways have not been established in murine mammary epithelial cells (MMECs), human breast epithelial cells or human carcinomas. In earlier studies, others and we using mouse mammary epithelial cells have established that activation of EGFR can promote survival in association with upregulation of Bcl-x_L a pro-survival family member of the Bcl-2 family. However, the mechanism(s) responsible for upregulation of Bcl-x_L is unknown.

In our recent studies [67] we have chosen pro-apoptotic, c-Myc-overexpressing MMECs derived from MMTV-c-Myc transgenic mouse tumors to address these issues. We demonstrated that EGF strongly activates both Akt and Erks over a similar timeframe, and that this activity is dependent of EGFR kinase activity. The importance of Akt and Erk1/Erk2 in EGF-mediated survival signaling in c-Myc-overexpressing cells was further confirmed through the finding that prolonged inhibition of PI-3K and MAPK/MEK activity leads to apoptosis. Apoptosis, induced by inhibition of PI-3K activity, was paralleled by down regulation of protein expression of both Akt and Erk1/Erk2. In addition, overexpression of constitutively activated Akt (Myr-Akt)

prevented c-Myc-mediated apoptosis, triggered by the inhibition of the EGFR tyrosine kinase activity. The mechanism through which Akt and possibly Erk promote survival in c-Myc-overexpressing cells appears to involve the upregulation of Bcl-x_L, suggesting that Bcl-x_L might be a novel target of Akt in MMECs (Figure 1). With the emerging role of activated EGFR family in human breast cancer, we believe it would be important to determine the anti-apoptotic targets of EGFR-stimulated Akt and Erk, in MMECs and human breast cancer cells. In support of our own *in vitro* studies, recent work by Hutchinson *et al.*, [68] directly assessed the role of Akt in mammary epithelial development and tumorigenesis, using transgenic mice. The results indicate that activated Akt provides a critical cell survival signal required for tumor progression, without causing metastatic progression.

Another very important molecules that play a role in mammary gland signaling are Stats, a transcription factors that are sequestered in the cytoplasm in an inactive form. Two members of the Stat family of transcription factors play a vital role in mouse mammary gland development. Stat5a was originally described as a regulator of milk protein gene expression and was subsequently shown to be essential for mammary development and lactogenesis. In contrast, Stat3 is an essential mediator of apoptosis and post-lactational regression. Other members of the Stat family may have specific, but as yet undemonstrated, functions in mammary development. However, since Stat1 activity is regulated during mammary development in a pattern different from Stats 3 and 5, this factor too may have a functional role. Phosphorylation by activated cytokines and growth factors receptors leads to dimerization and translocation to the nucleus where Stats can activate specific sets of genes [69]. Constitutive activation of Stats has been associated with invasive breast carcinomas and breast cancer cell lines but not with *in situ* carcinoma or benign lesions. Watson [70] postulates that cross talk between Stat pathways and

the ErbB family and Src may be important regulator of breast cancer progression.

Stats have been shown to have a binding site in the Bcl-x_L promoter region [71]; therefore when activated Stats as well could contribute to cellular survival through upregulation of this important pro-survival molecule. Recently, it was reported that STATs 1 and 3 expression in primary breast carcinomas correlate with EGFR, HER2, p53, ER, PR, p21/waf1, Bcl-x_L and Ki-67 expression [72].

III. SUMMARY AND FUTURE PROSPECTS

This chapter highlights the importance of some of the molecules in breast signaling. Clearly, the importance of signaling by the c-ErbB family, TGFβ/Smads, several other growth factors, PI-3K/Akt, MEK/Erk and Stats in mammary gland development and mammary tumorigenesis has been well documented. Despite that the recent insights revealed a significant amount of evidence for their role mostly by research using *in vitro* human cellular models and mouse models of human breast cancer, it appears that they are many more questions that remain unanswered. Indeed, studies to date have only started some of the fundamental questions of how the signaling molecules mentioned above contribute to physiological and pathological activities in the breast tissue. New and more sophisticated insights will undoubtedly provide a crucial information that will help understanding of the role of these molecules in breast cancer, and will help in providing valuable information for future therapies.

REFERENCES

1. Debies, M.T., and Welch, D.R. (2001). Genetic basis of human breast cancer metastasis. *J. Mammary Gland Biol. Neoplasia* **6,** 441-451.

2. Dickson, R.B., and Stancel, GM. (2000). Estrogen receptor-mediated processes in normal and cancer cells. *J. Natl. Cancer Inst. Monogr.* **27,** 135-145.

3. Haslam, S.Z., and Woodward,T.L. (2001). Reciprocal regulation of extracellular matrix proteins and ovarian steroid activity in the mammary gland. *Breast Cancer Res.* **3**(6), 365-372.

4. Kim, E.S., Khuri, F.R., and Herbst, R.S. (2001). Epidermal growth factor receptor biology (IMC-C225). *Curr. Opin. Oncol.* **13,** 506-513.

5. Prenzel, N., Zwick, E., Leserer, M., and Ullrich, A. (2000). Tyrosine kinase signaling in breast cancer. Epidermal growth factor receptor: convergence point for signal integration and diversification. *Breast Cancer Res.*, **2,** 184-190.

6. Adamson, E.D., Minchiotti, G., and Salomon, D.S. (2002). Cripto: a tumor growth factor and more. *J. Cell Physiol.* **190,** 267-278.

7. Salomon, D.S., Bianco, C., Ebert, A.D., Khan, N.I., De Santis, M., Normanno, N., Wechselberger, C., Seno, M., Williams, K., Sanicola, M., Foley, S., Gullick, W.J., and Persico, G. (2000). The EGF-CFC family: novel epidermal growth factor-related proteins in development and cancer. *Endocr Relat Cancer* **7,** 199-226.

8. Bianco, C., Adkins, H.B., Wechselberger, C., Seno, M., Normanno, N., De Luca, A., Sun, Y., Khan, N., Kenney ,N., Ebert, A., Williams, K.P., Sanicola, M., and Salomon;, D.S. (2002). Cripto-1 activates nodal- and ALK4-dependent and -independent signaling pathways in mammary epithelial cells. *Mol. Cell Biol.* **22,** 2586-2589.

9. Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis GD, *et al.* (1992). Identification of heregulin, a specific activator of p185erbB2. *Science* **256,**1205-1210.

10. Peles, E., Bacus, S.S., Koski, R.A., Lu, H.S., Wen, D., Ogden, S.G., Levy, R.B., and Yarden, Y. (1992). Isolation of the neu/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* **69,** 205-216.

11. Troyer, K.L., and Lee, D.C. (2001). Regulation of mouse mammary gland development and tumorigenesis by the ERBB signaling network. *J. Mammary Gland Biol. Neoplasia* **6,** 7-21.

12. Eccles, S.A. (2001). The role of c-erbB-2/HER2/neu in breast cancer progression and metastasis. *J. Mammary Gland Biol .Neoplasia* **6,** 393-406.

13. Luetteke, N.C., Qiu, T.H., Fenton, S.E., Troyer, K.L., Riedel, R.F., Chang, A., and Lee, D.C. (1999). Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* **26,** 2739-2750.

14. Sandgren, E.P., Schroeder, J.A., Qui, T.H., Palmiter, R.D., Brinster, R.L., and Lee, D.C. (1995). Inhibition of mammary gland involution is associated with transforming growth factor alpha but not c-myc-induced tumorigenesis in transgenic mice. *Cancer Res.* **55,** 3915-3927.

15. Schroeder, J.A., and Lee, D.C. (1998). Dynamic expression and activation of ERBB receptors in the developing mouse mammary gland. *Cell Growth Differ.* **9,** 451-464.

16. Dickson, R.B. and Lippman, M.E. (2000): "Autocrine and paracrine growth factors in the normal and neoplastic breast". *In* "Diseases of the Breast" 2nd ed, (Jay R. Harris), 303-317, Lippincott Williams & Wilkins, Philadelphia.

17. Amundadottir, L.T., and Leder, P. (1998). Signal transduction pathways activated and required for mammary carcinogenesis in response to specific oncogenes. *Oncogene* **16,** 737-746.

18. Amundadottir, L.T., Nass, S.J., Berchem, G.J., Johnson, M.D., and Dickson, R.B. (1996). Cooperation of TGF alpha and c-Myc in mouse mammary tumorigenesis: coordinated stimulation of growth and suppression of apoptosis. *Oncogene* **13,** 757-765.

19. Humphreys, R.C., and Hennighausen, L. (2000). Transforming growth factor alpha and mouse models of human breast cancer. *Oncogene* **19,** 1085-1091.

20. Schroeder, J.A., Troyer, K.L., and Lee, D.C. (2000). Cooperative induction of mammary tumorigenesis by TGFalpha and Wnts. *Oncogene* **19,** 3193-3199.

21. Humphreys, R.C., and Hennighausen, L. (1999). Signal transducer and activator of transcription 5a influences mammary epithelial cell survival and tumorigenesis. *Cell Growth Differ.* **10,** 685-694.

22. Liao, D.J., Natarajan, G., Deming, S.L., Jamerson, M.H., Johnson, M., Chepko, G. andDickson R.B. (2000). Cell cycle basis for the onset and progression of c-Myc-induced, TGFalpha-enhanced mouse mammary gland carcinogenesis. *Oncogene* **19,** 1307-1317.

22. Kenney, N.J., Smith, G.H., Johnson, M.D., et al., (1997). Cripto-1 activity in the intact and ovariectomized virgin mouse mammary gland. *Pathogenesis* **1,** 57.

23. Kenney, N.J., Smith, G.H., Rosenberg, K., Cutler, M.L., and Dickson, R.(1996). Induction of ductal morphogenesis and lobular hyperplasia by amphiregulin in the mouse mammary gland. *Cell Growth and Differ.* **7,** 1769-1781.

23. Amundattottir, L.T., Merlino, G.T., and Dickson, R.B. (1996). Transgenic models of breast cancer. *Breast Cancer Res. Treatment* **39,** 119-135.

24. Schroeder, J.A. and Lee, D.C. (1997). Transgenic mice reveals roles for TGF alpha and EGF receptor in mammary gland development and neoplasia. *Mammary Gland Biol. Neoplasia* **2,** 119-129.

27. Edwards, P.A.W. (1998). "Control of three dimensional growth pattern of mammary epithelium:role of genes of the WNT and erbB families studied using reconstituted epithelium". *In* "Mammary Development and Cancer" (Rudland, P.S., Fernig, D.G., Leinster, S., and Lunt, G.G.), 21-26, London, Portland Press.

28. Brandt, R. Eisenbrandt, R., Leenders, F., Zschiesche, W., Binas, B, Juergensen, C., and Theuring, F. (2000). Mammary gland specific hEGF receptor transgene expression induces neoplasia and inhibits differentiation. *Oncogene* **19,** 2129-2137.

29. Srinivasan, R., Poulsom, R., Hurst, H.C., and Gullick,W.J. (1998). Expression of the c-erbB-4/HER4 protein and mRNA in normal human fetal and adult tissues and in a survey of nine solid tumor types. *J. Pathol.* 185(3), 236-245.

30. Heldin, C.H., Miyazono, K., and Dijke, P.T. (1997). TGFβ signaling from cell membrane to nucleus through SMAD proteins. *Nature* **390,** 465-471.

31. Massague, J.(1992). Receptors for the TGF-beta family. *Cell* **69,** 1067-1070.

32. Ebner, R., Chen, R.H., Shum, L., Lawler, S., Zioncheck, T.F., Lee, A., Lopez, A.R., and Derynck, R. (1993). Cloning of a type I TGF-beta receptor and its effect on TGF-beta binding to the type II receptor. *Science* **260,**1344-1348.

33. Attisano, L., Carcamo, J., Ventura, F., Weis, F.M., Massague, J, and Wrana, J.L. (1993). Identification of human activin and TGF beta type I receptors that form heteromeric kinase complexes with type II receptors. *Cell* **75,** 671-680.

34. Liu, X., Yue, J., Frey, R.S., Zhu, Q., and Mulder, K.M. (1998). Transforming growth factor beta signaling through smad1 in human breast cancer cells. *Cancer Res.* **58,** 4752-4757.

35. McCune, B.K., Mullin, B.R., Flanders, K.C., Jaffurs, W.J., Mullen, L.T., and Sporn, M.B. (1992). Localization of transforming growth factor-beta isotypes in lesions of the human breast. *Hum. Pathol.* **23,** 13-20.

36. Robinson, S.D., Silberstein, G.B., Roberts, A.B., Flanders, K.C., and Daniel, C.W. (1991). Regulated expression and growth inhibitory effects of transforming growth factor-beta isoforms in mouse mammary gland development. *Development* **113,** 867-878.

37. Stampfer, M.R., Yaswen, P., Alhadeff, M., and Hosoda, J. (1993). TGF beta induction of extracellular matrix associated proteins in normal and transformed human mammary epithelial cells in culture is independent of growth effects. *J. Cell Physiol.* **155,** 210-221.

38. Wakefield, L.M., and Roberts, A.B. (2002). TGF-beta signaling: positive and negative effects on tumorigenesis. *Curr. Opin. Genet. Dev.* **12,** 22-29.

39. Xie, W., Mertens, J.C., Reiss, D.J., Rimm, D.L., Camp, R.L., Haffty, B.G., and Reiss, M. (2002). Alterations of Smad signaling in human breast carcinoma are associated with poor outcome: a tissue microarray study. *Cancer Res.* **62,** 497-505.

40. Ellis, M.J. (1999). " The insulin-like growth factor network and breast cancer". *In* "Breast cancer: molecular genetics, pathogenesis, and therapeutics" (Bowcock A, ed), 121. Totowa, NJ, Humana Press.

41. Bergstein, I., and Brown, A.M.C. (1999). WNT genes and breast cancer . *In* "Breast cancer: molecular genetics, pathogenesis, and therapeutics" (Bowcock A, ed), 181. Totowa, NJ, Humana Press.

42. Bronzert, D.A., Pantazis, P., Antoniades, H.N., Kasid, A., Davidson, N., Dickson, R.B., and Lippman, M.E. (1987). Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. *Proc. Natl. Acad. Sci. U S A* **84,** 5763-5767.

43. Kern, F.G. (1999). "The role of fibroblast growth factors in breast cancer pathology and progression". *In* "Breast cancer: molecular genetics, pathogenesis, and therapeutics" (Bowcock A, ed), 59. Totowa, NJ, Humana Press.

44. Ferrara, N., Houck, K., Jakeman, L., and Leung, D.W. (1992). Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocr. Rev.* **13,**18-32.

45. Wellstein, A., Fang, W.J., Khatri, A., Lu, Y., Swain, S.S., Dickson, R.B., Sasse, J., Riegel, A.T., and Lippman, M.E. (1992). A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. *J. Biol. Chem.* **267,** 2582-2587.

46. Rong, S., Bodescot, M., Blair, D., Dunn, J., Nakamura, T., Mizuno, K., Park, M., Chan, A, Aaronson, S., and Vande Woude, G.F. (1992). Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. *Mol. Cell. Biol.* **12,** 5152-5158.

47. Clevenger, C.V., Chang, W.P., Ngo, W., Pasha, T.L., Montone, K.T., and Tomaszewski, J.E. (1995). Expression of prolactin and prolactin receptor in human breast carcinoma. Evidence for an autocrine/paracrine loop. *Am. J. Pathol.* **146,** 695-705.

48. Bano, M., Kidwell, W.R., and Dickson, R.B. (1994). "MDGF-1: a multifunctional growth factor in human milk and human breast cancer". *In* "Mammary tumorigenesis and malignant progression" (Dickson, R.B. and Lippman, M.E., eds.), 193, Boston, Kluwer.

49. Roberts, R.A., James, N.H., and Cosulich, S.C. (2000). The role of protein kinase B and mitogen-activated protein kinase in epidermal growth factor and tumor necrosis factor alpha-mediated rat hepatocyte survival and apoptosis. *Hepatology* **31,** 420-427.

50. Sibilia, M., Fleischmann, A., Behrens, A., Stingl, L., Carroll, J., Watt, F.M., Schlessinger, J., and Wagner, E.F. (2000). The EGF receptor provides an essential survival signal for SOS-dependent skin tumor development. *Cell*, **10,** 211-220.

51. Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. *Curr. Opin. Cell Biol.*, **10,** 262-267.

52. Kandel, E.S., and Hay, N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp. Cell Res.* **253,** 210-29.

53. Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: a play in three Akts. *Genes Dev.* **13,** 2905-2927.

54. Coffer, P.J., Jin, J., and Woodgett, J.R. (1998). Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem. J.* **335,** 1-13.

55. Okano, J., Gaslightwala, I., Birnbaum, M.J., Rustgi, A.K., and Nakagawa, H. (2000). Akt/protein kinase B isoforms are differentially regulated by epidermal growth factor stimulation. *J. Biol. Chem.* **275,** 30934-30942.

56. Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **9,** 231-241.

57. Cardone, M.H., Roy N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S., and Reed JC. (1998). Regulation of cell death protease caspase-9 by phosphorylation. *Science* (Wash. DC) **282,**1318-1321.

58. Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96,** 857-868.

59. Jones, R.G., Parsons, M., Bonnard, M., Chan, V.S., Yeh, W.C., Woodgett, J.R., and Ohashi, P.S. (2000). Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels *in vivo. J. Exp. Med.* **191,** 1721-1734.

60. Pugazhenthi, S., Nesterova, A., Sable, C., Heidenreich, K.A., Boxer, L.M., Heasley, L.E., and Reusch, J.E. (2000). Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. *J. Biol. Chem.* **275,** 10761-10766.

61. Kuo, M.L., Chuang, S.E., Lin, M.T., and Yang, S.Y. (2001). The involvement of PI 3-K/Akt-dependent up-regulation of Mcl-1 in the prevention of apoptosis of Hep3B cells by interleukin-6. *Oncogene* **20,** 677-685.

62. Romashkova, J.A., and Makarov, S.S. (1999). NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* (Lond.) **401,** 86-90.

63. Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R.J., and Sonenshein, G.E. (2001). Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. *Oncogene* **20,** 1287-1299.

64. Moro, L., Venturino, M., Bozzo, C., Silengo, L., Altruda, F., Beguinot, L., Tarone, G., and Defilippi, P. (1998). Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *EMBO J.* **17,** 6622-6632.

65. Walker, F., Kato, A., Gonez, L.J., Hibbs, M.L., Pouliot, N., Levitzki, A., and Burgess. A.W. (1998). Activation of the Ras/mitogen-activated protein kinase pathway by kinase-defective epidermal growth factor receptors results in cell survival but not proliferation. *Mol. Cell Biol.* **18,** 7192-7204.

66. Hayakawa, J., Ohmichi, M., Kurachi, H., Kanda, Y., Hisamoto, K., Nishio, Y., Adachi, K., Tasaka, K., Kanzaki, T., and Murata, Y. (2000). Inhibition of BAD phosphorylation either at serine 112 via extracellular signal-regulated protein kinase cascade or at serine 136 via Akt cascade sensitizes human ovarian cancer cells to cisplatin. *Cancer Res.*, **60,** 5988-5994.

67. Ramljak, D., Coticchia, C.M., Nishanian, T.G., Saji, M., Ringel, M.D. Conzen, S.D., and Dickson, R.B. (2002). Epidermal growth factor inhibition of c-Myc-mediated apoptosis through Akt and Erk involves Bcl-x_L upregulation. *Oncogene (submitted).*

68. Hutchinson, J., Jin, J., Cardiff, R.D., Woodgett, J.R., Muller, W.J. (2001). Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression. *Mol. Cell. Biol.* **21,** 2203-2212.

69. Rane, S.G., and Reddy, E.P. (2002). JAKs, STATs and Src kinases in hematopoiesis. *Oncogene* **21,** 3334-3358.

70. Watson, C.J. (2001). Stat transcription factors in mammary gland development and tumorigenesis. *J. Mammary Gland Biol. Neoplasia* **6,** 115-127.

71. de Groot, R.P., Raaijmakers, J.A., Lammers, J.W., and Koenderman, L. (2000). STAT5-Dependent CyclinD1 and Bcl-xL expression in Bcr-Abl-transformed cells. *Mol. Cell Biol. Res.* Commun. **3,** 299-305.

72. Berclaz, G., Altermatt, H.J., Rohrbach, V., Siragusa, A., Dreher, E., and Smith, P.D. 2001). EGFR dependent expression of STAT3 (but not STAT1) in breast cancer. *Int. J. Oncol.* **19,** 1155-1160.

Table 1. Members of the epidermal growth factor receptor (EGFR) family and their ligands

Receptor	Ligands
EGFR (c-erb-B1)	Epidermal growth factor (EGF), Transforming growth factor-α, Amphiregulin, Heparin-binding EGF-like growth factor, Betacellulin, Epiregulin
c-erb-B2 (HER2)	Not established
c-Erb-B3 (HER3)	Neuregulins
c-Erb-B4 (HER4)	Neuregulins

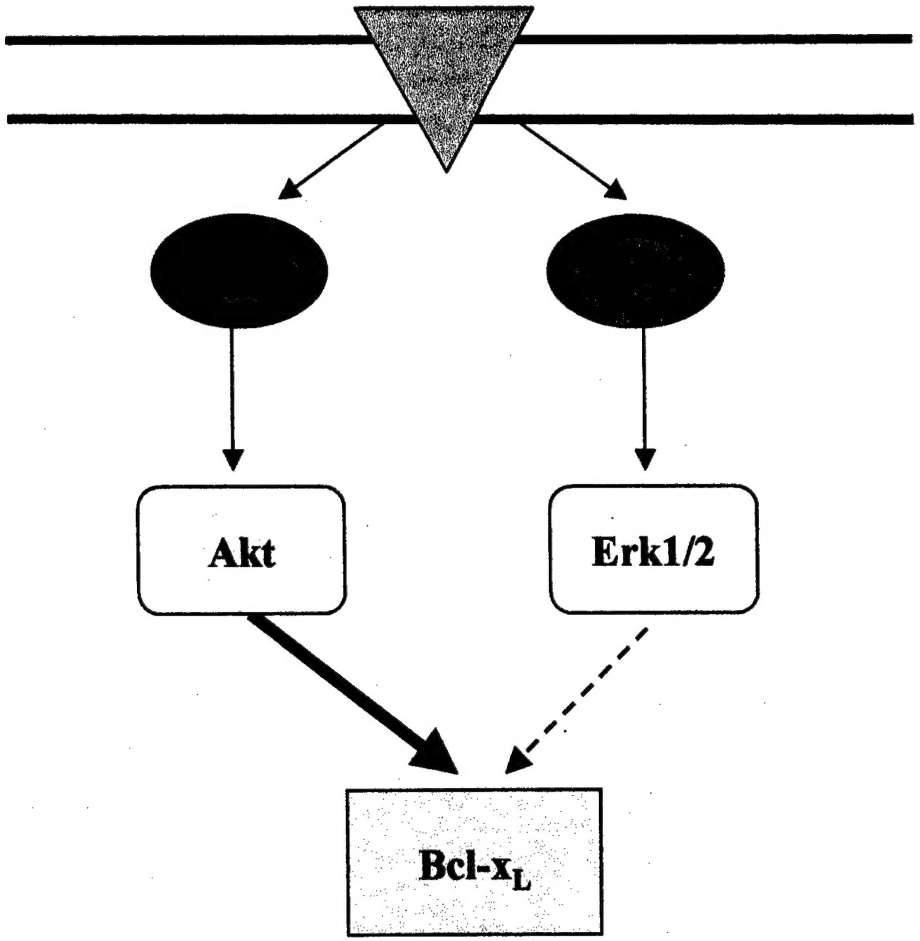
The four ErbB receptors are shown along with their ligands. Note that ErbB2 does not have a direct binding ligand.

Table 2. A diverse group of growth factors and other molecules thought to play paracrine role in breast cancer

Signaling molecules	Cellular response
Insulin like growth factors (Type I and II)	Stimulatory/Tumor cell
Wnt growth factor family	Stimulatory/Tumor cell
Platelet-derived growth factors A and B	Stimulatory/Tumor cell
Fibroblast growth factors (FGF)	Stimulatory/Tumor cell
Vascular endothelial growth factor (VEGF)	Angiogenesis/Tumor cell
Hepatocyte growth factor (HGF), receptor c-Met	Stimulatory/Tumor cell
Prolactin	Stimulatory/Tumor cell
Mammary-derived growth factor 1 (MDGF-1)	Inhibitory/Tumor cell
Pleiotrophin (developmental neurotropic factor)	Stimulatory/Tumor cell

Proposed autocrine factors in breast cancer. Tumor cell release variety of growth factors might play autocrine roles *in vivo* (this is based mostly on their activity *in vitro*). Several of the same factors are known to play paracrine and endocrine roles as well.

Figure 1. Proposed model of Bcl-x_L regulation by Akt and possibly Erk. Activated Akt upregulates Bcl-x_L protein by presently unknown mechanism in mouse mammary epithelial cells overexpressing c-Myc. This might lead to inhibition of apoptosis.


Growth factors
Akt
Erk1/2
Bcl-x_L